LYSOZYME

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by

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INTRODUCTION

Lysozyme is a bacterolytic enzyme which is found in most animal tissues and secretions and whose substrate is the mucopolysaccharide of the bacterial cell wall. Because of its specificity and its distribution, there is some evidence (27) that lysozyme plays an important role in the intracellular resistance to bacteria; however, the bactericidal role of lysozyme in the body has not received good supporting evidence except for Micrococcus lysodeikitus (8) where inhibition of intracellular killing was effected by the use of the methyl ester of lysozyme, a competitive inhibitor.

It was felt that better evidence concerning the role of lysozyme in resistance to infection could be obtained if a specific inhibitor of lysozyme were found, and, thus, an antibody to rat kidney lysozyme was prepared. Once the anti-lysozyme antibody was obtained, it was decided to study the distribution of lysozyme within phagocytic cells in preparation of attempts to inhibit the intracellular killing of bacteria.

Even though the kidney is not a member of the reticuloendothelial system, a high concentration of lysozyme is found (81). In order to determine the function of this kidney lysozyme, it was decided to study the distribution of lysozyme within the kidney.

All of these studies were carried out with antibody to rat kidney lysozyme and with rat and mouse tissues, using fluorescent antibody techniques. However, there were difficulties in obtaining enough material for the extraction procedure and in obtaining large quantities of rat

polymorphonuclear leucocytes. Because of these difficulties and because of the interest in human resistance to infection, it seemed fruitful to continue these studies with lysozyme from human kidney and with human tissues; therefore, it was necessary to establish an extraction procedure for lysozyme from human kidneys.

Recently the separation of isoenzymes has become a diagnostic aid in clinical medicine. Since it has been shown by chromatographic procedures (124, 125) that there do exist isoenzymes of lysozyme, it seemed appropriate to try to electrophoretically separate the isoenzymes to see if this separation would be of any diagnostic aid.

THE HISTORY OF LYSOZYME

Fleming's Work

In 1922 Alexander Fleming reported (30) the discovery of a substance in nasal secretions from a patient with acute corzya which was able to lyse certain strains of bacteria. He called this substance, lysozyme. The substance was discovered because the patient's nasals secretions, when cultured, grew out only an occasional staphylococcus instead of the expected lush growth for the first three days while on the fourth day they grew out a bacterium which Fleming named Micrococcus lysodeikitus. This bacterium was extremely susceptable to lysis by lysozyme, and Fleming used this property to establish a test system for the presence of lysozyme (32). A well would be made in an agar plate and filled with agar in which the solution or tissue to be studied had been placed. The whole plate would then be covered by a layer of agar containing M. lysodeikitus which would then grow up except in a zone around the test well where there would be lysis if lysozyme were present. The greatest dilution at which a zone of lysis was still present was taken as the titre of the lysozyme in the substance. With this method Fleming showed that human tears had the highest lysozyme concentration followed by nasal secretions and sputum. Florey (35) using the same method but a more complete list of sources, showed that the greatest titres were found in the kidney, stomach, and salivary glands followed by the spleen, lungs, liver, and cartiledge.

Although Fleming was primarily a bacterologist, he did a remarkable job in characterizing some of the physical properties of lysozyme (31). He showed that its size (molecular weight) was approximately equal to that of trypsin by agar diffusion studies. He also demonstrated that lytic activity increased with temperatures up to 60°C and that both acid and base inhibited the rate of the reaction. The optimal NaCl concentration for the lysis of M. lysodeikitus was 0.5% (33). Contrary to most enzymes the activity of lysozyme remained after boiling in acetic acid for five minutes. He also noted that either cotton or filter paper absorbed lysozyme. Fleming felt that lysozyme was neither an antibody nor an opsomin because it was not consumed during the course of the reaction.

Ultimately Fleming and Allison noted that occasionally colonies grew in the inhibitory zones. These strains were lysozyme resistant. Since a strain that was resistant to a lysozyme from one source was resistant to the action of all lysozymes, Fleming at first felt that lysozyme from all sources was the same (34). In later papers, however, he adopted the view that lysozymes from different sources were not identical.

Besides his work on the action of lysozyme on M. lysodeikitus, Fleming studied the effect of lysozyme on other bacteria: 75% of laboratory bacteria were lysised by a 1/1000 dilution of tears, 7 of 8 strains pathogenic for animals but not for man, and 72% of intestinal streptococci but no strains of salmonella or pneumococcus.

Other Contributors

Other investigators during the 1910's and 1920's had done work on what was later realized to be lysozyme. Laschtschenko (61) and Rittger and Sperry (101) showed that egg white had a bactericidal effect that was

destroyed by heating. The spectrum of this bactericidal effect was the same as that described by Fleming for lysozyme from human tears. These investigators believed, however, that the bactericidal power was a function of egg white only.

In 1921 Gengou (40) reported the presence of a bactericidal substance in extracts from white blood cells which had many of the properties that Fleming had described for lysozyme. At the same time Turro (128) reported the existance of a bacterolytic substance in tissues.

In one of his papers Fleming quoted Melchnikoff, "Nature does not make use of antiseptics to protect the skin and mucous membranes. The fluids which moisten the surface of the mouth and of other mucous membranes are not microbicidal". After Fleming had shown the mistruth of this statement in relation to M. lysodeikitus, other investigators began to study the effect of human secretions on more pathogenic bacteria. Ridley (98), using 90% tears, studied a variety of bacteria showing that at that concentration the tears inhibited many strains of staphylococcus, streptococcus (hemolytic and fecal), and pneumococcus. Whereas 90% tears did inhibit these bacteria, 75% did not. Ridley showed that in many conditions in which infections of the eye were common (phlyclinular conjunctivitis, interstitial keratitis, foreign body retention, and epiphora), the lysozyme content was only 50% of normal; at this concentration of lysozyme many bacteria which were usually inhibited were able to grow, and Ridley felt that the lowered lysozyme concentration was the cause of the increased infection rate. Fleming thought as a result of Ridley's work that one possible explanation of the presence of intracellular gonococci was that the white cells in which

the gonococci were found were different in lysozyme content since the gonococcus was readily destroyed in phagocytic experiments.

Two other investigators, Rosenthal and Lieberman (104), were interested in the effect of the lysozyme in milk (both human and cow) on the intestinal flora of infants. Human milk has a much higher concentration of lysozyme than cow's milk probably due to the increased volume of the cow's production. When either boiled human or cow's milk was fed to infants having equal numbers of Gram negative and positive organisms, there was an increase in the Gram negative population whereas, if the infants were fed human milk, there was a decrease in the number of Gram negative organisms.

Thus, before the advent of antibiotics, much of the knowledge concerning a bactericidal agent, lysozyme, had been discovered. However, with the emergence of other means of controlling infectious disease, interest in lysozyme as a drug diminished. Later it was replaced by interest in lysozyme as a microbiological research tool.

PHYSICAL PROPERTIES OF LYSOZYME

So many substances have been discovered that can cause the lysis of M. lysodeikitus that certain properties have to be possessed by a lytic enzyme in order for it to be called, lysozyme; it has to be a basic protein of low molecular weight which retains its activity after boiling in acetic acid for 1-2 minutes and whose lysis of a suspension of M. lysodeikitus produces the appropriate degradation products (52). In order to give lysozyme a more exacting name it has also been called β-glucosaminidase, murmidase, N-acetylmuramide glycanohydrolase or E.C.3.2.1.17. These names all relate to the nature of its specific substrate.

Lytic enzymes which fulfill these criteria are found in the tissues and secretions of most humans and in many other vertibrates and invertibrates. They also are found in plants (papaya latex)(117), associated with phage (T_2)(57), and in bacteria (B. subtilis)(97). Most studies on lysozyme, however, have been done with egg white lysozyme. The molecular weight of all strains of lsyozyme studied have been in the range, 14,500 ± 500 with the exception of papaya latex lysozyme whose molecular weight is 25,000. The molecular weight of egg white lysozyme from its amino acid analysis is 14,386. The shape of the native protein is probably an oblate ellipsoid with an axial ratio of 3:1 and with a hydration of 0.6 grams of water per gram of protein (21, 62, 122).

The complete amino acid sequence of egg white lysozyme recently has been elucidated separately by both Jolles (55) and Canfield (11). (Figure 1) Lysozyme is the first protein which has been shown to contain all the amino acids. The large numbers of basic amino acids in the

molecule are responsible for its high isoelectric point, 10.5-11.0. There are four S-S bridges, the reduction of which produce only one peptide chain showing that the molecule is only a single chain.

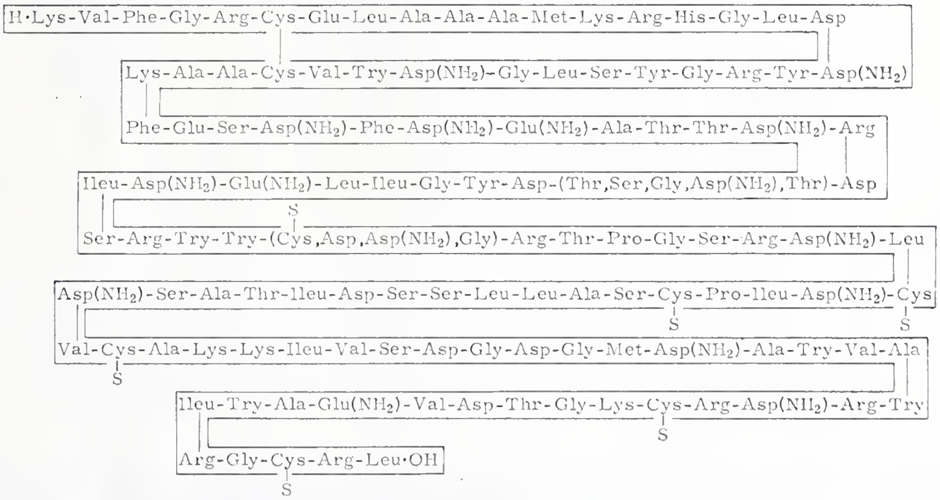


Figure 1

Amino Acid Sequence of Egg White Lysozyme

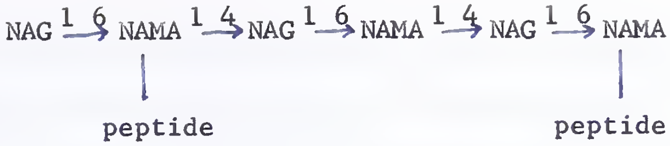
The determination of the amino acid sequence of lysozymes from different sources is being attempted in order to discover if there are any areas of the sequence that are identical in the different lysozymes. These areas of agreement would probably be the active site and the combining site. However, thus far no areas of identity have been found.

ACTION OF LYSOZYME

It was not until 1936 that attention was turned from the lysis of sensitive organsims to the elucidation of the characteristics of the substrate for the enzyme. In that year Meyer and his co-workers (73) showed that the substrate for lysozyme probably was a mucopeptide in the cell wall of the bacteria. Then Epstein and Chain (28) published their work in which they stated that the substrate for lysozyme was a high molecular weight polysaccharide which liberated N-acetylated aminodexoses upon degradation. They felt that in the susceptable bacteria the polysaccharide was found in a water insoluble form in the cell wall and that after reaction with lysozyme it became water soluble.

The definitive work on the nature of the substrate for lysozyme has been done by Salton (105, 107, 108, 110, 113, 114). He has shown that the products of <u>M. lysodeikitus</u> cell wall digestion by lysozyme consist of two fractions, one dialysiable and one not dialysiable. The non-dialysiable fraction has a molecular weight of 10,000 to 20,000. The structure of the dialysiable fraction has been determined to be 6-0-β-N-acetylglucosaminyl-N-acetylmuramic acid and its dimer with a β $(1 \rightarrow 4)$ bond. It seems, therefore, that the backbone of the mucopeptide molecule contains N-acetylglucosamine and N-acetylmuramic acid bound in pairs with alternating β $(1 \rightarrow 4)$ lysozyme sensitive and β $(1 \rightarrow 6)$ lysozyme resistant bonds. To this backbone would then be coupled the various other components of the cell wall.

The dialysiable fraction contains di- and tetra-saccharides. In view of the recent evidence that lysozyme can act as a condensing as well as a cleaving enzyme, the presence of dimers is not surprising.



$$\text{NAG}\xrightarrow{1\;6}\underset{\substack{|\\ \text{peptide}}}{\text{NAMA}}\xrightarrow{1\;4}\text{NAG}\xrightarrow{1\;6}\text{NAMA}\xrightarrow{1\;4}\text{NAG}\xrightarrow{1\;6}\underset{\substack{|\\ \text{peptide}}}{\text{NAMA}}$$

Figure II

Polysaccharide of Bacterial Cell Wall

All cells studied that are susceptable to lysozyme lysis at least three amino acids have been found: D-glutamic acid, alanine and either lysine or diaminopimelic acid (139). These amino acids are arranged in peptide chains that are joined to the muramic acid residues. The existance of large molecular weight, non-dialysiable digestion fragments is possibly due to crosslinkages between the peptide chains so that, although the lysozyme sensitive bonds would have been cleaved, the molecule would still be held together by the crosslinkages (112).

The cell walls of lysozyme sensitive M. lysodeikitus is almost pure substrate for lysozyme and a good source for substrate for enzymatic studies. However, as Fleming first showed, there are strains of M. lysodeikitus that are resistant to the action of lysozyme. Brumfitt, Warlaw, and Park (9) have shown that in one resistant strain (L250) the resistance is associated with increased 0-acetyl content of the polysaccharide. If the acetyl groups are removed by hydrolysis, the susceptability of the organism to lysis is restored. However, not all lysozyme resistance can be correlated with increased 0-acetyl content(69).

As Fleming and Ridley showed, there is a wide range of Gram positive organisms that are sensitive to lysozyme, including strains of staphylococcus, pneumococcus, hemolytic and fecal streptococcus, gonococcus, and salmonella. Most Gram positive organisms, however, are resistant (138). If the cell walls of lysozyme resistant bacteria are chemically altered, however, then they may become sensitive to the action of lysozyme; thus, the cell walls

from Group C streptococci become sensitive after trypsinization (60) and those from Group A streptococci after hot formamide extraction (59). Protoplasts can be formed from Group D streptococci by the action of lysozyme (7).

Since Gram positive cell walls are primarily mucopeptide in nature, containing peptides, amino sugars, and sugar constituents, and, since the chemical constituents are the same for resistant and non-resistant organisms, except in exceptional cases, chemical differences do not seem to be the basis of resistance. It has been suggested that resistance may be due to difference in the bonds between the amino sugars, increased crosslinkages between the peptide chains, or decreased sugar content. All of these would decrease the amount of substrate readily available to the lysozyme and, thus, decrease the sensitivity of the cell wall to the action of lysozyme (109).

As a group Gram negative bacteria are less sensitive to lysozyme than Gram positive ones (83); however, some organisms like Brucella abortus are moderately lysed (106). In contrast to the relatively simple structure of the Gram positive cell wall, the walls of Gram negative bacteria are most complex, containing polysaccharides, a greater range of amino acids, and lipids.

Treatment of Gram negative organisms may produce forms that are lysozyme sensitive. Pseudomonas aeruginosa after treatment with acetone becomes lysozyme sensitive (131). The treatment of Gram negative organisms with Versine (EDTA) can lead to lysozyme lysis in a tris buffer system (95). Incubation of Gram negative bacteria at low pH, $\sim$3.5, followed by sudden alkalization to a basic pH, $\sim$10.0, will lead to lysis after lysozyme treatment. Ralston and Elberg (90,91) have shown that the addition of glycine

to the culture media of polymorphonuclear leucocytes will increase the intracellular killing of Brucella. Although it has not been definitely proven, it seems that all these treatments increase the availability of the substrate to the action of the enzyme. Acetone, heat and acid will destroy the outer layer of the cell wall while the glycine and EDTA will chelate inhibitory metal ions that are found in the cell wall.

Another interesting experiment is that of Weidel who showed that the lytic enzyme of the T_2 phage, which is a lysozyme, can lysis the inner most cell wall layer of E. coli without any pretreatment (58, 133, 134). This observation probably explains how spontaneous lysis of the infected bacteria takes place. Since the lysozyme is already within the cell and since the polysaccharide is the inner most layer, there is no need for adjuvants in order for the lysozyme to reach the substrate.

It is possible that within the system of human bacterial defense mechanisms lysozyme is the final agent in the process of bacterial destruction (43). Whereas, when a bacterium is first injested by a member of the rectuloendothelial system, it may be resistant to the action of lysozyme; the action of the other enzymes within the cell (trypsin, acid and alkaline phosphatases) may alter the structure of the cell wall in such a manner that the wall is then lysozyme sensitive.

Glynn and Brumfitt (8) have shown that in the case of Micrococcus lysodeikitus its intracellular destruction can be inhibited by the methyl ester of lysozyme, which is a specific competitive inhibitor of lysozyme; these experiments have increased the evidence for the role of lysozyme in the body's defense mechanisms.

ASSAY METHOD OF LYSOZYME

History

The first method for the determination of lysozyme content was that of Fleming in which the greatest dilution of a substance that caused the production of an area of lysis on an agar plate seeded with _M. lysodeikitus_ was the titre of the lysozyme present. This original method as well as the other methods which evolved involved the lysis of suspensions of _M. lysodeikitus_ had the limitation that they gave only qualitive lysozyme concentrations.

The most quantative method that has been devised for lysozyme determinations is that of Litwack (63,86). In his test system to a standard suspension of _M. lysodeikitus_ in standard buffer is added the solution to be tested, diluted with buffer if needed. The transmissance of the solution is plotted against time, and then taking the rate of change the concentration is determined from a standard curve.

There has been much disagreement as to the nature of the reaction between lysozyme and the cell walls of _M. lysodeikitus_. Various authors have reported the order of the reaction as $0^{\underline{st}}$, $1^{\underline{st}}$, or $2^{\underline{nd}}$ with respect to the enzyme. Litwack showed that in his system that a straight line is produced if 1/absorbence is plotted against time for the first four minutes of the reaction, and, thus, during this period the reaction is $2^{\underline{nd}}$ order and that then the order becomes $0^{\underline{st}}$ or $1^{\underline{st}}$ if the reaction is allowed to progress.

In preliminary experiments it was shown that the plot of transmissance vs time over the first two minutes of the reaction gave a straight line and that the plot of the slope of the reaction against

the concentration of the enzyme in the range, 0 — 10 ug/ml, gave a linear relationship.

Method

A test suspension of M. lysodeikitus, strain L2, 28 mgm%, is made up in a buffer that is 90% phosphate buffer, pH 6.5, 0.1 M and 10% 1% NaCl. Readings are taken on a Spekker (Helger and Watts, Ltd., London) with a H508 filter using a 2x5 cm, curved bottom cell. To 4.5 ml. of the test suspension of the bacteria is added 0.5 ml of the unknown solution with vigorous pipetting. The transmissance of the solution with 5.0 ml of the test suspension as a blank is read and plotted at 15 second intervals for two minutes. The slope is determined, and the lysozyme content read from the standard curve. A standard curve has to be prepared for each day using crude Armour egg white lysozyme (Armour and Co., Chicago) as the reference material since the ambient temperature and the condition of the test suspension will alter the standard curve.

Discussion

The Micrococcus lysodeikitus is grown on large nutrient agar plates, harvested, and freezed dried. When the test suspension is first made up, it will have an OD of 60 which will decrease over the first 24 hours due to spontaneous lysis. Good results are obtained if the OD is between 50 and 60; if it is any lower, the change is the transmissance will not be great enough to give good results.

Figure 3 shows the typical graph of transmissance against time; there is a sudden increase in transmissance with the addition of the unknown solution, followed by a period of steady change, and finally with high lysozyme concentration a slowing of the rate of change due to product inhibition of the reaction.

Figure 4 shows a typical standard curve of the rate of change of the transmissance of the test bacterial suspension against the lysozyme concentration. The nature of the standard curve, which is linear at low lysozyme concentrations, changes depending upon the age of the bacterial suspension. A new suspension has a negative y intercept showing its relative resistance to lysis while an old suspension has a positive y intercept and a great slope showing is greated susceptibility to lysis.

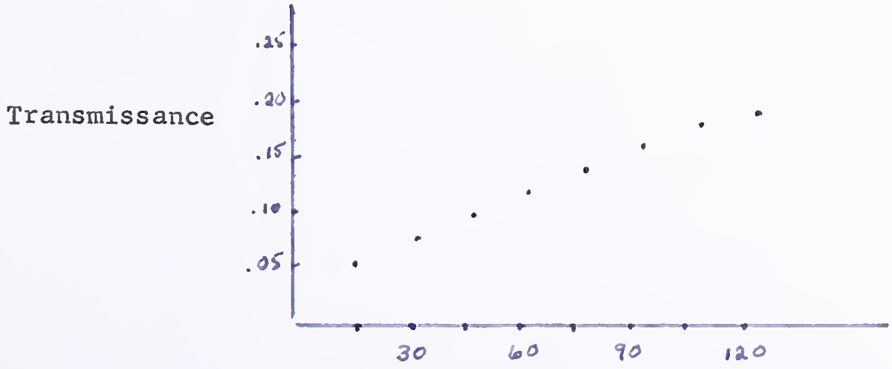


Figure 3

Transmissance of bacterial suspension vs time

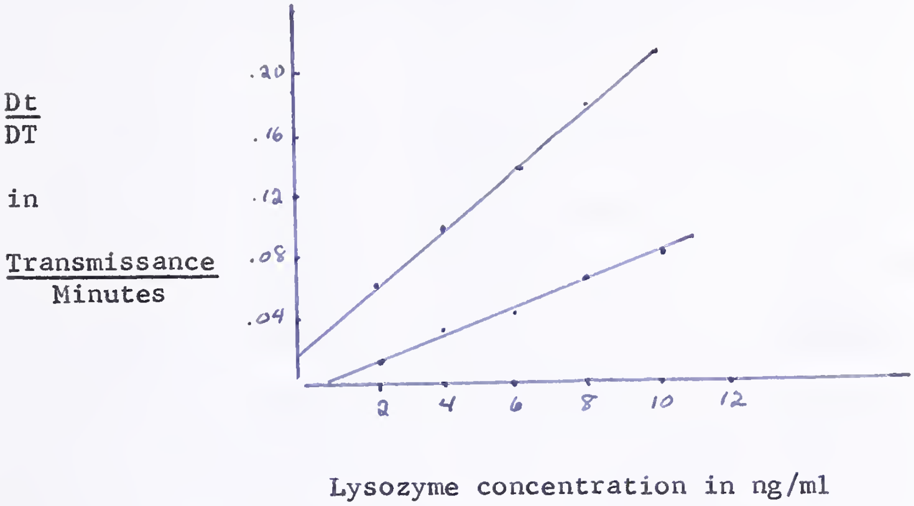


Figure 4

Rate of change of transmissance vs lysozyme concentration

PREPARATION OF HUMAN KIDNEY LYSOZYME

History

The richest readily available source of lysozyme is hen's egg white, and most of the original attempts to purify lysozyme delt with this source. Wolff in 1927 (137) attempted to remove inert protein from egg white with colloidal iron and then to precipitate the lysozyme with acetone. This method was followed by a series of methods (3, 4, 96, 102), the most commonly used of which was that of Meyer (72) in which the acetone precipitated proteins were extracted with acid-alcohol, followed by alcohol precipitation, isoelectric precipitation of active proteins by sulfuric acid, and then precipitation of the lysozyme by flavianic acid.

The most recent of the precipitation methods is that of Perri in which the organ under study is homogenized with three volumes of M/15, pH 6.5, phosphate buffer, and then an equal amount of 1% acetic acid-alcohol added. The mixture is centrifuged, the supernatient mixed with six volumes of absolute alcohol, the precipitate saved, redissolved in water, and recrystallized from an alcohol-mixture.

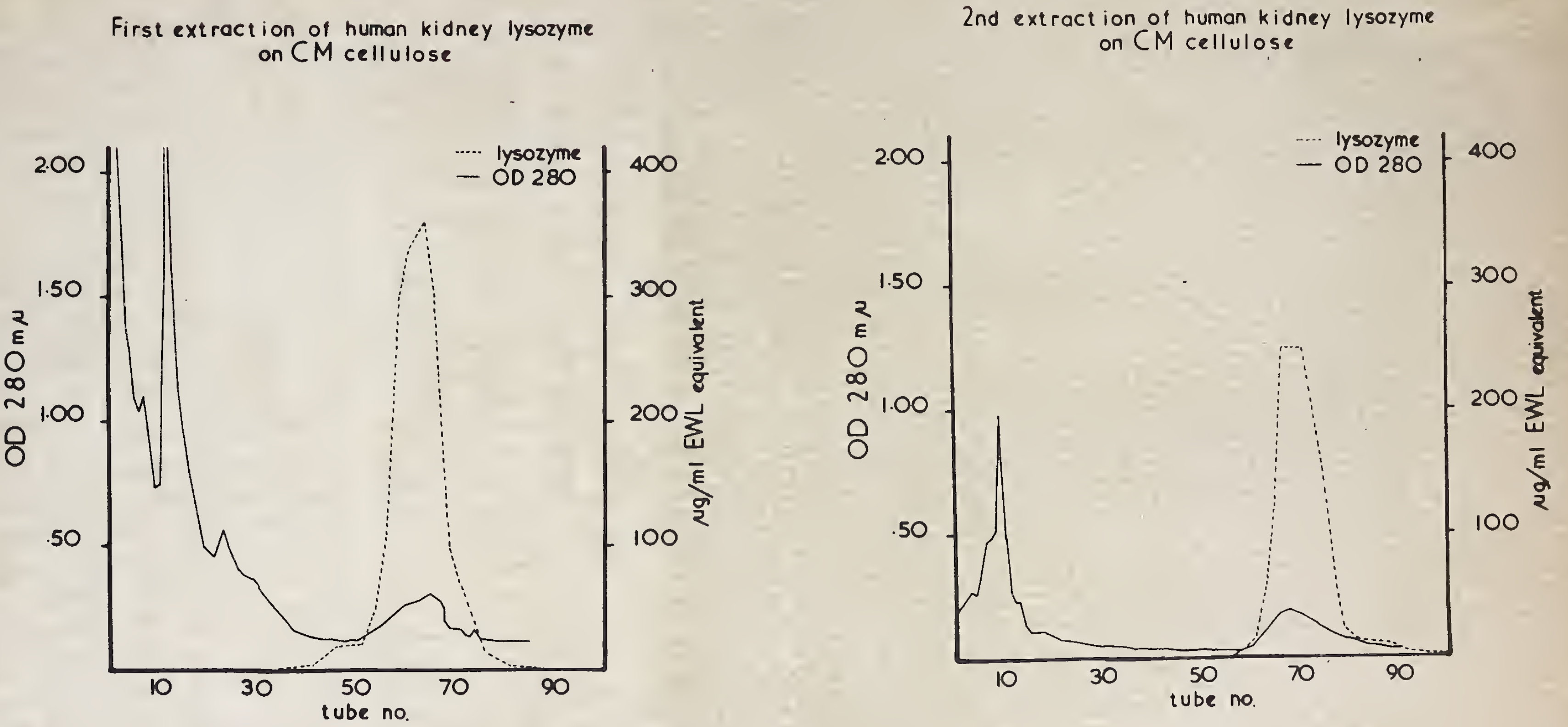
With the emergence of ion exchange column chromatography an easier way to purify the enzyme using a weakly acid resin, IRC-50, was shown. Reports followed of the isolation of lysozyme from rabbit spleen, dog kidney, dog spleen, rat kidney, and papaya latex. The first reported isolation of a lysozyme from a human source was that of Litwack (68) concerning the isolation of lysozyme from human kidneys; Jolles then followed with the report of the isolation from human milk (53).

When it was decided to prepare a pure lysozyme from a human source, the kidney was chosen because of its high lysozyme content and ready availability. In all reported methods for the isolation of lysozyme, the resin, IRC-50, was used. However, in preliminary attempts to reproduce the methods of Litwack, Jolles, and Perri, great difficulty was encountered in eluting the enzyme from the resin. Therefore, it was found necessary to devise a new extraction procedure.

Method

> Post-mortum human kidneys are homogenized in three volumes of distilled water, and then three volumes of 5% acetic acid-alcohol are added. The mixture is stored in the cold, solid material is removed by centrifugation, the supernatient saved, and its pH adjusted to 5.5. The solution is run on to a column of carboxymethylcellulose (Whatman's CM 70, London, England), 1x20 cm, which is prepared to the following specifications: phosphate buffer, 0.01 M, pH 5.5. The column then is eluted with a pH-molar gradient. A 500 ml mixing chamber is filled with phosphate buffer, 0.01 M, pH 5.5, to which is attached a resevoir which contains phosphate buffer, 0.2 M, pH 7.5, so that a constant volume is maintained in the mixing chamber.

Ten milliliter samples are collected with a flow rate of 60 ml/hr. Protein concentrations are determined by absorbency at 280 mu, and lysozyme concentration determined in the usual manner. All tubes with activity greater than 10 ug EEWL/ml are pooled, acidified to pH 5.5,



Purification of human kidney lysozyme using a pH and molar gradient:

phosphate buffer, 0.01M, pH 5.5 - 0.2M, pH 7.5, reservoir, 500 cc.

Figure 5

and dialysed against distilled water for one hour. The solution then is run on to a second CMC column prepared as the first and again eluted with the same pH-molar gradiant. All tubes with activity greater than 10 ug EEWL/ml are pooled, dialysed against distilled water for three hours, and freezed dried.

Results

Other authors have noted that during the purification of human kidney lysozyme a 25% increase in activity was noted which lead to the hypothesis that an inhibitor of lysozyme may be naturally present. During the purification procedure which has been outlined above, an even more marked increase in activity is noted.

Table I

Total lysozyme activity during purification

	Total lysozyme in mg equivalent EWL
Filtered supernatient	30
After 1st elution from CMC	44
After 1 hour dialysis	35
After 2nd elution from CMC	95
After 3 hour dialysis and freeze drying	67

The first elution produces a 50% increase in activity which is of the same magnitude as has previously been reported. However, the second elution produces a 200% increase in activity. The actual increase may be higher since there are absolute losses which can better be seen when small amounts are used. It is probable that there is a substance in kidney which is inhibitory for lysozyme, either specifically or non-specifically, and which is removed by the purification procedure.

Since lysozyme has such a high isoelectric point, it was reasonable to assume that the inhibitor had a lower isoeletric point and, therefore, either would not be absorbed on to the CMC at all or would be eluted before the lysozyme. Attempts to discover the location of the inhibitor by mixing various elution samples with purified lysozyme produced no inhibition. At this point an observation helped to clarify the situation. In one preparation of human kidney lysozyme there was no activity in the filtered supernatient before it was run through the CMC column. If the activity was measured after it had been run through the CMC column, there was lysozyme activity present. This observation was surprising in that the column was obviously overloaded, and the theory that the inhibitor was more acidic than lysozyme would mean that the lysozyme would be more readily bound than the inhibitor; the converse, however, seemed to be true, that the inhibitor was more readily bound than the lysozyme and, therefore, must be more basic. Since lysozyme has an isoelectric point of 10.5, there are not many naturally occuring compounds that have a higher isoelectric point.

One physologically occuring compound which fulfills some of these properties is spermine (48, 123), a biological polyamine which is found throughout the body. Hirsh and Dubos studied its effect on the tubercle bacilli, especially its ability to inhibit growth. During the extraction of spermine they noted that lysozyme activity was present at all stages of the procedure and could finally be removed by dialysis although spermine's molecular weight is 203 and lysozyme's is 14,500. They were interested in obtaining pure preparations of spermine, not lysozyme, and therefore, it is impossible to know if the spermine had any inhibitory effect on the lysozyme. However, spermine is a basic compound with the

same distribution as lysozyme that is extracted with lysozyme; these are all characteristic of the postulated inhibitor.

Discussion

Post-mortum kidneys are obtained from persons who have not had clinical evidence of renal disease and kept frozen until used, 1 to 6 months. It seems that more lysozyme is extractable from the kidneys which have been frozen for the longest length of time although it has not been proven.

In the above method distilled water is used instead of 15/M phosphate buffer is order to keep the salt concentration as low as possible to facilitate the adsorption on to the CMC. The 50% alcohol-water precipitates most of the larger molecular weight proteins while the acidification of the solution makes the lysozyme more stable. Some difficulty was encountered due to shrinkage of the column because of the high alcohol content of the mixture, and for this reason CM-Sephadex which was first used was discarded in favor of CMC.

The adjustment of the pH of the supernatient to 5.5 causes a precipitate to form which clogs the CMC column if used immediately. Therefore, the adjusted supernatient is allowed to stand overnight and filter through filter paper. According to the studies of Fleming this filtration will lead to the loss of some lysozyme, but this source of loss was not studied.

In some preparations a peak appears between the first elution peak and the lysozyme peak which is pink in color and is probably the heme group of cytochrome c, liberated by the acid-alcohol, with an absorbance peak at 428 mu.

The gravest difficulty that is encountered is the problem of desalting. It is necessary to reduce the salt concentration of the elutent from the first column before it is run on to the second column. Also it is necessary to desalt the final sample as much as possible before freeze drying in order to minimize protein inactivation. Usually to desalt protein solutions, visking dialysis tubing is used. However, due to is peculiar properties, lysozyme has the ability to diffuse through regular dialysis tubing. If the data of Leonis is correct and lysozyme has an axial ratio of 5:1, then the cross-sectional area of the molecule might be small enough to pass through the pores of the tubing. There are several methods reported to decrease the permeability of dialysis tubing including baking, stretching, and acetylation. Usually 24/32" tubing was used although according to Craig the 18/32" diameter tubing is the least porous, but there is no explanation of this difference (25).

Tubing that is heated at 90°C for 4 hours is decreased 10% in length and becomes colored with a brown material that is removed by washing; however, the rate of loss of lysozyme from the heated tubing is not reduced as compared to controls. Craig also has shown that permeability can be decreased by the longitudinal stretching of the dialysis tubing. Craig thinks the stretching changes the spacial configuration of the pores. By using a rack the length of the tubing could be increased 20% and held overnight in the stretched form. When tubing thus prepared is tested with lysozyme dissolved in water with unstretched tubing as a control, the overnight loss is only 25% of the control tubing; however, if the lysozyme is dissolved in M/15 phosphate buffer, there is no difference in the rate of loss. Thus, the presence of salt during the dialysis produces such a force on the lysozyme that there is no difference in the

rates of diffusion. Attempts at acetylation of the tubing with acetic anhydride in pyridine did not produce a tubing with diffusion properties for lysozyme significantly different from the controls.

Another method of desalting is the use of Sephadex of an appropriate size to exchange buffer for distilled water (84). Sephadex, however, is not completely inert so that in low salt concentrations because of its weakly acidic charge basic proteins like lysozyme stick to the column (41). Thus, when attempts are made to remove the salt, the lysozyme sticks and is lost. A further possibility is to exchange the phosphate buffer for a volatile buffer (54), such as pyridine acetate, which can be removed during the freeze drying process. Attempts at exchanging buffers were a success; however, since it takes 14 grams of Sephadex to completely exchange 10 ml of solution, it is an impractical method when liters have to be desalted.

RABBIT ANTI-KIDNEY LYSOZYME

History

The first reported preparation of an anti-serum to lysozyme was that of Jermoljewa and Bujanowskajo (49) who in 1931 prepared anti-serum to amorphous egg white lysozyme, thus, showing that lysozyme was antigenic. In 1937 Roberts reported that anti-serum to egg white lysozyme did not react with lysozyme from human or cat saliva. These results were the first direct evidence that there was more than one lysozyme (103).

The first preparation of an anti-serum to crystallized lysozyme was in 1947 by Smolens and Chainy (119). They showed that the lytic activity in their anti-serum was due to rabbit lysozyme that was not inhibited by the anti-egg white lysozyme. The rabbit lysozyme could be removed from the anti-serum by bentonite absorption. The best work on the nature of the antibodies to lysozyme is a series of papers by Shinho, Fujio et al. (38, 39, 115). They showed that in their anti-serum to EWL there were two specific neutralizing antibodies, one directed against the combining site and one against the active site.

Immunization schedule

For the primary injections 10 mg of protein is emulsified in 4 ml of complete Freund's adjuvant and is injected intramuscularly into each limb of an adult rabbit. After one month the animal is boosted with alum precipitated antigen, 4 mg, that is prepared by precipitating an aluminum potassium phosphate-protein solution with NaOH (74). This precipitate is washed with saline and injected in four intramuscular

sites in a 1:1:2 ratio on three alternate days. Bleeding is carried out via the marginal ear vein. The blood is allowed to clot, refrigerated, spun, and the serum is removed, absorbed with bentonite, filtered, and stored at 4°C.

Cross reactivity

The first studies on cross reactivity of different lysozymes and their derivitives were carried out using horse anti-egg white lysozyme kindly donated by Professor R. R. Porter. These studies were done utilizing the double diffusion technique of Ouchterlony (76,77). Attempts to use commercial agar for the gel met with little success since the lysozyme tended to bind to the agar and not to diffuse; thus, acrylamide was used as the gel base in barbitone buffer, pH 8.6, I=0.07. The gel is poured into Petri dishes to a depth of 1 cm. Since the acrylamide will not gel in the presence of O_2, the Petri dishes are placed in anerobic jars, evacuated, filled with hydrogen, and allowed to gel. This method produces good results, and the dishes are placed in the refrigerator until used. A hexangonal pattern with 1 cm wells and 2.5 cm centers is used for the studies.

The solution of antigen and antibody are diluted with buffer so that the precipitate line are midway between the wells. Studies were carried out with egg white lysozyme (EWL), methylated egg white lysozyme (ML), rat kidney lysozyme (RKL), rat spleen lysozyme (RSL), mouse kidney lysozyme (MKL), mouse spleen lysozyme (MSL), rat polymorph lysozyme (RPL), and mouse polymorph lysozyme (MPL). When horse anti-egg white lysozyme was used, there was cross reactivity between EWL and ML with spur formation but no reactivity with RKL, MKL, RSL, or MSL (Figure 6).

Figure 6

Gel diffusion of anti-EWL and various lysozymes

Figure 7

Gel diffusion of anti-RKL and various lysozymes

An anti-serum was prepared to rat kidney lysozyme purified by the method of Perri (80). With this anti-serum there was cross-reactivity between the lysozymes from rat and mouse sources but no reaction with lysozyme from egg white. There was an inconsistant double line against rat spleen and polymorph lysozyme but only one line against rat kidney lysozyme and lysozyme from all mouse sources (Figure 7).

The cause of the double line against RSL and RPL could not be determined. Since on immunoelectrophoresis the two lines move together and since they have come through a complicated purification procedure together, the double line is due to either a variant of lysozyme (either isoenzymes, dimer or denatured enzyme) or a closely related substance whose nature is unknown.

These experiments show that antigenically several distinct forms of lysozyme exist with the antigenic differences becoming less as the phyllogenetic differences become less. It has been shown by other authors that lysozymes from closely related species have antigenically similar lysozymes.

γ-globulin preparation

In fluorescent antibody work it is possible to work with whole serum, but the best results are obtainable if the γ-globulin fraction of the serum is used. The classical method of γ-globulin preparation is the use of its insolubility in high salt concentrations (56).

> Anhydrous $NaSO_4$ is added to the serum to make an 18% solution, and the precipitate is removed by centrifugation and then redissolved in water. This protein solution is then made up to 12% with respect to $NaSO_4$, and the precipitate is collected and dissolved. The γ-globulin is again precipitated by

12% $NaSO_4$, redissolved, dialysed against buffered saline, and freezed dried.

The yield of this method is 10 mg/ml of γ-globulin from normal rabbit serum. On electrophoresis a small amount of albumin contaminate is seen but no globulins other than gamma (Plate 1).

Another method of purification of γ-globulin from whole rabbit serum has been developed from the fact that γ-globulins are the most basic of the serum proteins (120, 121).

A DEAE-Sephadex column is prepared and equiliberated against phosphate buffer, pH 6.4, 0.0175 M. Usually one gram of DEAE-Sephadex is used for 3 ml of serum. The serum is dialysed against the buffer overnight, run on to the column, and eluted with the same buffer. The γ-globulin is eluted as soon as the hold-up volume comes through, and all other serum proteins are bound to the column. All samples with O.D.s above 0.50 are collected and pressure dialysed to a suitable concentration.

The yield of γ-globulin by this method is 4 mg/ml which is electrophoretically pure.

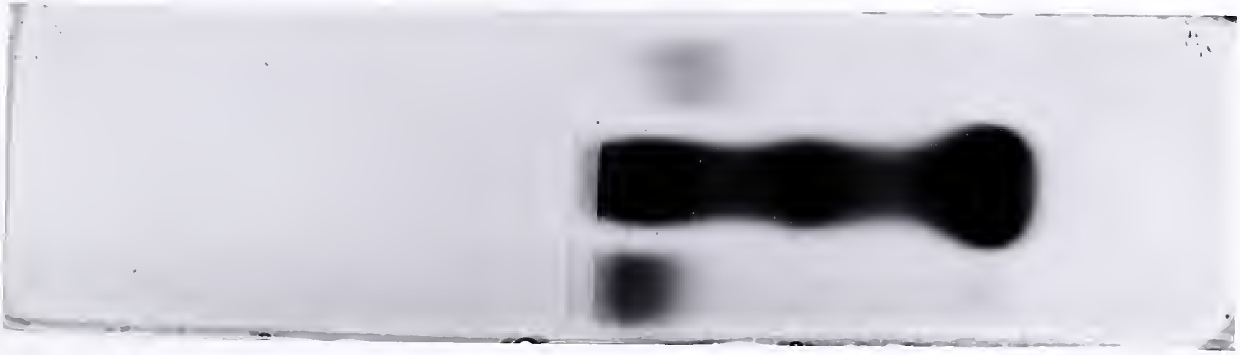


Plate 1

Electrophoresis in 5% acrylamide, barbitone buffer, pH 8.6, I=.07, 18 hours

Top: Fluorescein labelled γ-globulin

Middle: Whole rabbit serum

Bottom: $NaSO_4$ precipitated γ-globulin

FLUORESCENT LABELLED ANTIBODY

Introduction

Once the specific nature of the antigen-antibody reaction was known, it was logical that this specificity would be used to establish the location of antigens. The most successful method using this specificity is that of the fluorescent labelled antibody. In 1942 Coons showed that an antibody molecule could be conjugated with a fluorochrome with little loss of either activity or specificity; the titre of the labelled serum is 30% less than the whole serum.

The three principal fluorochromes that are used are fluorescein isothiocynate (FITC), Lissamine Rhodamine B (RB 200), and 1-dimethylaminophthalene-5-sulfonic acid (DANS). The work in these studies was done with the first two of these. Aminofluorescein is a derivative of 4-nitrophthalic acid and resorcenol which absorbs maximally at 495 mu and emits at 515 mu (85). Originally the isocynate was used for conjugation but more recently the isothiocynate has been shown to be more usable since it does not hydrolyse as readily (99,100). The emission peak of FITC labelled proteins used in fluorescent microscopy is the one at 520 mu which is excited by light at 325 mu. Lissamine Rhodamine B absorbs maximally at 570 mu emits at 595 mu. The sulphonyl chloride is used for the conjugation procedure, and the conjugated proteins used in fluorescent microscopy absorb at 435 mu and 350 mu and emit at 595 mu (yellow) and 710 mu (red) respectively. Thus, the shorter the wavelength of the exciting light, the higher the wavelength of the emitted light, or more red. Since both emission peaks are usually excited, the resultant color is orangiest. RB 200 is especially

useful when the tissue under study has a high yellow or green autofluorescence which would make contrast with fluorescence difficult.

Method

(1) The classical method of labelling proteins with FITC is that of Coons (24) with modifications.

The method is as follows:

> The γ-globulin solution is made up to a concentration of 5 mg/ml using Na_2CO_3-$NaHCO_3$ buffer, pH 9.0, 0.5 M. The amount of FITC is calculated on the basis of 3-4 mg FITC/gm γ-globulin; the crystalline form of the fluorochrome with twice the activity of the amorphous form is used. The FITC is made up in cold dry acetone, 1 mg/ml. The protein solution is placed in a cold room in a flask with a magnetic stirrer, and the FITC added dropwise over a 15 minute period. The mixture is allowed to react overnight with constant stirring and with care that the pH is kept constant by the addition of buffer.
>
> The reaction mixture is then run through a G-50 Sephadex column that has been equilibrated with buffered saline. The fluorescent labelled protein comes off as a rapidly moving band while the unreacted fluorescein is a slower moving band. Sephadex removes the unreacted fluorochrome and exchanges buffers. If needed, the solution can be concentrated by pressure dialysis.

The ratio of fluorochrome molecules to protein molecules (F/P ratio) of fluorescein labelled protein prepared by this method is 1:75:1.

(2) Another method of preparing fluorescein labelled protein takes a much shorter time, 30 minutes (94). Celite is a silicous earth on which chemicals can be absorbed. A commercial preparation (Calbiochem, Los Angeles) is available that is 10% FITC by weight. If this preparation is mixed with a protein solution, the rate of reaction is more rapid than when both reactants are in solution. The reaction has gone to completion within one minute of the Celite and the protein solution mixing.

The method is as follows:

> The γ-globulin solution is made up to a concentration of 10 mg/ml with Na_2CO_3-$NaHCO_3$ buffer, pH 9.0, 0.5 M. The amount of 10% FITC added is calculated on the basis of 2 mg FITC/gm protein. The Celite-FITC complex is added to the protein solution in a test tube and shaken vigorously for three minutes. The Celite is removed by centrifugation, and the supernatient is placed on a G-50 Sephadex column and eluted with buffered saline.

The F/P ratio of fluorescent antibody prepared by this method is 3:3:1.

(3) Although the F/P ratio of the fluorescent antibody labelled by the Celite method is quite good, the distribution curve of the F/P ratios is very broad. This means that many antibody molecules are overlabelled or underlabelled as compared to the average ratio. A new method of labelling procedures a population of fluorescent antibody molecules which are much more homogenous in their F/P ratio and which have a high F/P ratio(13).

The method is as follows:

> The protein is made up to 10 mg/ml with Na_2CO_3-$NaHCO_3$ buffer, 0.025 M, and placed in 8/32" dialysis tubing.

> This protein solution is dialysed against at least 10 volumes of the same buffer with 15 mg% of FITC added at 4°C with continuous stirring. The extent of the labelling is dependent upon how long the reaction is allowed to run.

Because the FITC solution is weak and the rate of the reaction is slow, the degree of labelling is very uniform. The amount of non-specific staining is very low even with high F/P ratios, 5— 10:1. The unreacted FITC is removed from the labelled protein by elution from a G-50 Sephadex column with buffered saline. If the reaction is allowed to run for 64 hours, the F/P ratio is 5.8:1 while a run of 96 hours will produce a ratio of 10.5:1.

(4) RB 200 labelled antibody is prepared by means of the Celite method using Celite with 5% RB 200. The amount of RB 200 to be used is calculated on the basis of 50 mg/gm γ-globulin. The procedure used is the same as in the Celite-FITC method. The F/P ratio of this method is 2.5:1.

Protein Determination

The easiest method of protein concentration determination is the spectrophotometric reading of a protein solution at 280 mu (6) which is the absorbence peak for tyrosine. For γ-globulin the conversion factor is 1.4 O.D. units/mg/ml protein. However, this method is not usable for the determination of lysozyme concentrations or for the determination of the protein concentrations of fluorescent antibody solutions. Spectrophotometric methods (127, 130) for the determination of lysozyme protein concentration fail because of the high tryptophan content in lysozyme. Several methods are reported to avoid this difficulty, but all attempts to obtain constant conversion factors have failed. Because of the strong

absorbency of the fluorochromes in the region below 300 mu attempts to quantitate the fluorescent protein solution by means of spectrophotometric methods were inaccurate.

The method that was adopted was that of Folin-Lowry (70) in which the protein concentration is expressed in terms of a reference curve of bovine serum albumin. Both Folin phenol reagent and $CuSO_4$-Na tartrate are used as reagents. The Folin phenol reagent reacts with aromatic amino acids while the $CuSO_4$-Na tartrate reacts with the peptide bonds giving an intrinsic double measurement.

The procedure is as follows:

> Equal parts of 1% $CuSO_4$ and 2% Na tartrate are diluted 1:50 in 2% Na_2CO_3 in 0.1 N NaOH. 0.5 ml of the protein solution is added to 2 ml. of the test reagent, shaken vigorously, and allowed to sit for 10 minutes. After 0.2 ml of Folin reagent is added with vigorous shaking, the solution is allowed to sit for 30 minutes. Readings taken at 750 mu and 550 mu then are used to determine protein concentration from the standard curve for BSA.

The amount of protein detected by this method is 10 — 100 ug/ml, far less than is needed for a Biuret determination. The lysozyme values obtained by this method are very constant, and the fluorochromes exert no interfering effect.

FLUORESCENT ANTIBODY STAINING

Direct Technique

Introduction

The Coons fluorochrome antibody labelling technique has been refined, but the basic principals have remained the same (23). The gamma globulin from a specifically immune serum is isolated and conjugated with a fluorochrome. The labelled antibody then is applied to the section or smear under study and allowed to react. The unattached antibody is removed by washing, and the slide then is viewed by means of ultraviolet light microscopy. The areas of specific fluoroescence represent the sites of localization of the antigen-antibody complex.

Method

After preparation of the fluorescent antibody it is stored at 4°C. If a large amount of anti-serum has been prepared, it is best to divide it into small vials and store at -20°C. When the anti-serum is needed, it is brought to room temperature and adsorbed with pig liver powder (100mg/ml) that has been moistened with buffered saline, pH 7.1, to reduce losses. The anti-serum should be adsorbed twice, including once overnight in the cold. The liver powder is removed by high speed (30,000 rpm) centrifugation for 45 minutes. The centrifugation also

removes denatured proteins which are produces during the freezing and thawing which, therefore, should be kept at a minimum.

The fluorescent antibody is applied to cover the specimen that has previously been bathed in buffered saline to produce favorable conditions for the antigen-antibody reaction. The slides are placed in a moist chamber at 37°C for 30-45 minutes if the protein concentration of the fluorescent antibody is 3-5 mg/ml. If the concentration is less, 1 mg/ml, then 1-1½ hours may be necessary. The slides then are washed for 30 minutes with at least three changes of buffered saline. Prolongation of the washing time greatly enhances histologic definition. The sections are allowed to dry and then are mounted in 50% glycerol-50% buffered saline. The usual 90% glycerol is not used as a mounting medium because of its inherently high degree of non-specific fluorescence.

Discussion

The controls that are necessary with the direct staining technique are primarily two. The slides are pre-incubated with either unconjugated immune γ-globulin or normal γ-globulin. There should be a diminution or abolition of the specific staining on the slide that was incubated with the immune γ-globulin since it "blocks" by combining with the antigenic sites. There should be no change in the specific staining of the slide that was pre-incubated with the normal γ-globulin. If there is any diminution, it is due to non-specific protein adsorption

rather than the blockage of specific antigenic sites. If a corresponding fluorescent antibody is available against an antigen not under study, the slide should be stained with it. There should be no staining since any staining will be due to the non-specific attachment of the fluorescent labelled protein.

The chief advantage of the direct technique, thus named because the fluorochrome is directly attached to the specific immune antibody, is that the staining for more than one antigen is possible if the different antibody molecules are labelled with different fluorochromes. For instance, it would be possible to stain for the presence of bacteria within phagocytic vacuole while staining for the vacuole also. The fluorochromes can be chosen so that they can be visualized with the same incident light or under different conditions.

The chief difficulty of the direct technique is that the number of fluorochrome molecules that are attached to the antigen under study is about one-third of that possible with the indirect technique; therefore, the specific staining is fainter. Complete abolition of staining in the "blocking" control is almost impossible since it requires only a few non-blocked antigenic sites to produce some staining; thus, producing a diminution of specific staining rather than abolition.

Indirect Technique

Introduction

In 1945 Coombs published (22) his work that is the basis of the indirect fluorescent antibody technique. The process is a two step one: the immune γ-globulin is used in an unconjugated form, and, after it has reacted with the antigen, a fluorescent antibody whose specificity is against the globulin of the animal species from which the immune globulin

was obtained is applied and allowed to react. Thus, the fluorochrome is attached to the antigen through two separate antigen-antibody complexes. Usually since the immune globulin is obtained from rabbits, the fluorescent labelled globulin is sheep anti-rabbit globulin which is commercially available.

Method

The fluorescent labelled anti-rabbit globulin, immune γ-globulin, and normal γ-globulin are adsorbed twice with pig liver powder (100 mg/ml), once overnight in the cold, before the first use and once before subsequent use. The liver powder and denatured protein are removed by high speed (30,000 rpm) centrifugation for 45 minutes. The immune globulin, 3-5 mg/ml, is applied to the section or smear that has previously been equilibrated with buffered saline and allowed to react in a moist chamber at 37°C for 45 minutes. The slides then are washed for 15-30 minutes in 2-3 changes of buffered saline. The slides are allowed to dry to keep from diluting the fluorescent labelled anti-rabbit globulin which is then applied. The slides are incubated in a moist chamber at 37°C for 45 minutes, washed for 30-60 minutes with 3-4 changes of buffered saline, allowed to dry, mounted in 50% glycerol-50% buffered saline, and viewed under ultraviolet light microscopy.

Discussion

The controls that are used with the indirect method are the use of normal γ-globulin or buffered saline instead of the immune γ-globulin. Both of these controls should be negative. To whatever degree the normal γ-globulin is positive is the degree of "non-specific" staining that is due to adsorption of the rabbit globulin onto the specimen. The degree of positive staining on the slide stained with only fluorescent antibody is the "non-specific" staining due to non-specific reactions of the fluorescent labelled protein.

The principal advantage of the indirect method is the increase in specific fluorescence that is possible. However many antigenic sites there are on the rabbit γ-globulin molecule, it is by that factor that the number of fluorochrome molecules is greater in the indirect as compared to the direct method. Also the controls present a much more "all or none" picture than the direct control slides. The chief liability of this method is that, except with great difficulty, it is impossible to stain for more than one antigen at a time.

Non-specific Staining

The chief difficulty in fluorescent antibody staining is the non-specific adherence of protein molecules to the tissue under study. The reason for this adsorption is that the protein molecules are charged, and, if the charge difference between the globulin molecule and the components of the specimen is great enough, then the strong forces of attraction will prevent antibody removed through washing. The more an antibody molecule becomes conjugated (the greater its F/P ratio), the more positively charged it becomes (36), and the more likely it will be non-specifically adsorbed. Thus, if the electrophoresis of normal γ-globulin is compared to fluorescent labelled γ-globulin, (Plate 1)

it is seen that the fluorescent labelled globulin migrates further because of its greater positivity (26).

Goldstein (45) has shown that samples with low F/P ratios, 2-3.5, give high specific staining and low non-specific staining while samples with high F/P ratios, 5-10, give high non-specific staining. It can thus be seen how the adsorption of the fluorescent antibody solution with pig liver powder decreases non-specific staining; the highly charged molecules are allowed to react non-specifically with the tissue powder and, thus, decrease the ultimate non-specific staining of the tissues under study. The best tissue powder to use, if a soluble antigen is under study, is a powder made from the tissue under study. If the antigen is not soluble, the powder of an organ from the same species that does not contain the antigen is best. If neither of these tissue powders is available, then pig liver powder can be used. The organ is homogenized and then washed with saline until all the blood is removed. The powder then is washed with acetone until all the color is removed. After drying the powder is ready for use.

Goldstein claims that the best way to eliminate non-specific staining is to fractionate the fluorescent antibody on DEAE and to use only those fractions that have a suitable F/P ratio, 2-3.5. Cebra (12) has taken this method one step further by pointing out that, although antibodies with high F/P ratios are undesirable, molecules with very low F/P ratios are equally undesirable. Since they are poorly conjugated, they effectively block antigenic sites without producing fluorescence. Cebra feels that in Goldstein's method it is not recognized that there is a variation in the charge of normal γ-globulin. Thus, in one of Goldstein's fractions with a good F/P ratio, there will be some relatively positively charged γ-globulin with a low F/P ratio and some relatively negatively charged

γ-globulin with a high F/P ratio. Both of these would produce a fluorescent labelled antibody with the same charge and a solution with an acceptable F/P ratio.

Cebra belives that the γ-globulin first should be fractionated into several samples by using stepwise elution from DEAE with a weak phosphate buffer and increasing ionic strength, thus, producing samples of increasing acidity. Each sample then would be conjugated separately and separated on DEAE into samples whose F/P ratios would be determined. Thus, instead of two variables, the degree of conjugation plus the charge of the original γ-globulin, there would be only one variable, the degree of conjugation. Cebra has obtained good specific staining with little non-specific staining with fluorescent protein solutions as low as 200 ug/ml. These separation methods can be used for the fluorescent antibody in either the direct or indirect methods.

Microscopy

For all the fluorescent antibody work a Reichert microscope, "Zetopan" model, with an Osram HBO 200 mercury vapor lamp, was used. For brightfield viewing a BG 12/6 mm excitation filter was used which transmits 45% of the light at its maximum of 420 mu and has an excitation range of 350-475 mu. The cuf-off filters used for brightfield viewing were GG 9/1 mm and OG 1/1.5 mm. These filters were yellow in color and were acceptable with fluorescein whose emmission is at 495 mu; however, they did distort the normal blue auto-fluorescense of the tissues producing a green auto-fluorescence.

This filter system was found to be unacceptable for the RB 200 labelled antibody. Whereas usually RB 200 emits primarily at 715 mu and secondarily at 595 mu, in these experiments maximal fluorescence

was obtained at 595 mu which necessitated the use of darkground microscopy to that sufficient incident UV light could be used to stimulate the 595 mu emission of the RB 200. An UG 1/1.5 mm excitation filter was used which because of its thinness transmits 80% at its maximum of 360 mu, and a colorless GG 13/3 mm, Wartin 2 B, was used as the cut-off filter which allowed blue auto-fluorescence. The 715 mu emission of RB 200 was distinguished by using a darkground setup with a BG 12/2 mm excitation filter and a GG 9/1 mm cut-off filter. It was shown that the red and white fluorescence coincided although there was greater definition with the white fluorescence.

MACROPHAGES AND POLYMORPHONUCLEAR LEUCOCYTES

Experimental

Peritoneal exudate macrophages were produced by the injection of 5 ml of 1% glycerin intraperitoneally four days prior to the date of harvest. The peritoneum of the animal was washed out with Hank's solution to which 10% inactivated calf serum and 100 units/ml of heparin had been added. The usual yield per rat was 2 x 10^7 cells of which greater than 90% were macrophages on stained smear. The cell suspension was placed in plastic wells, 1 x 2 cm, on chemically cleaned slides, the slides were placed in a moist chamber, and the cells were allowed to settle onto the slides. The wells were then removed, the slides washed in buffered saline, and the cells were flashed dried with a jet of CO_2 and fixed in cold absolute methenol.

In some animals the trauma of the harvesting of the cells would injure the gastrointestinal tract, thus contaminate the macrophage suspension with bacteria. In other experiments dead bacteria were injected intraperitoneally prior to the harvesting of the cells.

Polymorphonuclear leucocytes were prepared in one of two ways. They were obtained from either the blood of rats or mice or acute peritoneal exudates from the same animals. Blood could be collected from the plureal space of an animal after opening the chest wall and cutting the pulmonary artery. The blood was collected in heparinized pipettes, then was placed in plastic wells on clean slides, and was treated as the macrophage suspension is. Polymorphonuclear leucocytes could also be obtained by the production of an acute peritoneal exudate. Five milliliters of nutrient broth is injected intraperitoneally 2-4 hours prior to harvest. The cells

are harvested in Hank's solution with 10% inactivated calf's serum and 100 units/ml of heparin added and fixed as before. The cells obtained were up to 50% polymorphonuclear leucocytes. In some experiments dead bacteria are injected prior to harvest.

Results

Normal rat peritoneal macrophages studied by the indirect fluorescent antibody technique using anti-rat kidney lysozyme showed a diffuse even staining of the cytoplasm with no staining of the nucleus (Plate 3-4). In some phagocytic experiments discrete and well circumscribed areas of cytoplasmic staining were observed while the cell remained generally even staining.

The appearance of the polymorphonuclear leucocytes is much different from that of the macrophages. They characteristically show a pattern of discrete highly fluorescent cytoplasmic granules with a lighter, more diffuse background(Plate 2). When bacteria are added, a great variety of staining patterns are seen including cells with only a few large granules, cells with "crescent" formation, and cells with intense diffuse staining (Plate 5-10).

Discussion

The probable explanation of the differences in the staining patterns of the polymorphonuclear leucocytes and the macrophages and in the changes that occur during and after phagocytosis is found in the work of Cohn, Morse and Hirsh (14-20). They have studied the distribution of hydrolytic enzymes within phagocytic cells in both the resting state and after phagocytosis.

They first studied polymorphonuclear leucocytes from acute rabbit peritoneal exudates which had been lysed by hypertonic sucrose. The

resultant lysate then was separated by differental centrifugation into three parts: 400g pellet (nuclear material and cell walls), 8200g pellet (larger formed elements of the granulocyte including granules), and supernatient (smaller formed elements plus the cytoplasmic contents). They then assayed each of the fractions for the activity of acid and alkaline phosphatase, ribonuclease, deoxyribonuclease, β-glucuronidase, cathepsin, and lysozyme. For all enzymes except lysozyme and cathepsin, at least 75% of the activity was found associated with the 8200g pellet with the rest being equally distributed between the nuclear and supernatient fractions (15). For cathepsin and lysozyme the activity of the granular fraction was greatly decreased: 25%-nuclear, 45%-granular, 30%-supernatient. The activity of the nuclear fraction probably is due to lysozyme contamination of the nuclear DNA. There probably is no lysozyme in the nuclear fraction.

Hirsh and Cohn then showed that after phagocytosis the polymorphonuclear leucocytes became degranulated. This result was easily studied in rabbit polymorphs because of the large size of their granules. The proximity of a granule to a phagocytic vacuole seemed to determine which ganules disappeared because the granules nearest to the vacuoles disappeared first (17, 47). The question arose as to whether the granules were liberating their contents into the cytoplasm or into the phagocytic vacuole. Cohn believed that the contents were released into the vacuoles based upon the electron micrographs of Luck (14). The distribution of enzymes after phagocytosis and degranulation was studied. It was found that in all enzymes studied before there was a change in the distribution from being primarily in the granular fraction to being in the supernatient fraction (16). In all cases over 60% of the activity was found in the supernatient.

Cohn and Weiner also subjected macrophages (peritoneal exudates, alveolar, and BCG stimulated alveolar) to the same analysis. In terms of the total activity of any one enzyme there was no difference in the enzyme activity regardless of source except for lysozyme (19). Whereas 10^6 peritoneal exudate macrophages had the activity of 0.4 ug of egg white lysozyme, normal alveolar macrophaged had 3.2, and BCG stimulated alveolar macrophaged had 9.2. In these studies the centrifugal fractions were a 500g pellet (nuclei and cell walls), a 15,000g pellet (granular including mitochondria), and a supernatient fraction. Again the enzymes, acid phosphatase, cathepsin, β -glucuronidase, and lysozyme, were studied. In all cases greater than 70% of the activity was associated with the granular fraction. Moreover, it was shown by density equilibrium studies that the granules containing the hydrolytic enzymes, including lysozyme, were more dense than mitochondria. After phagocytosis when the assay was repeated, there was an increase in the supernatient activity of the enzymes. Three hours after phagocytosis 50% of the lysozyme activity was found in the supernatient fraction (20). When the macrophages were stained for acid phosphatase before phagocytosis, a fine, even, discrete particulate pattern was noted throughout the macrophage. After phagocytosis there was a loss of particulate cytoplasmic staining with an accumulation of the enzyme (acid phosphatase) around the injested particle. This accumulation of the enzyme around the injested particle seemed to suggest that the degranulation of the granules released their contents into the phagocytic vacuole.

The distribution of lysozyme as shown by fluorescent antibody (44) localization agrees well with the enzymatic analysis of Cohn and his co-workers. In the polymorphonuclear leucocytes the staining is equally distributed between the granules and the cytoplasm while in the macrophages

it is seen diffusely throughout the cytoplasm. When the polymorphs and macrophages are stained with Giesma's stain, the polymorphs show the presence of granules while the macrophages do not. However, the staining of the macrophages for enzymatic activity (acid phosphatase) shows a fine granular pattern.

The localization of lysozyme in vacuoles in the polymorphonuclear leucocytes after phagocytosis is an interesting finding. Originally it had been hypothesized that the lysozyme would be found diffusely throughout the cytoplasm after degranulation; however, after the results were seen, it was observed that, instead of a more diffuse staining pattern, there was a more particulate pattern, large areas of staining which seemed to coalesce. The question then arose as to whether the areas of the localization of the lysozyme were the phagocytic vacuoles in which the phagocytosed bacteria were and where the apparent destruction of the bacteria takes place. In an attempt to show that the areas of increased lysozyme concentration were indeed the phagocytic vacuoles, double fluorescent antibody studies were undertaken. It was attempted to stain the lysozyme (with fluorescein labelled antibody) and the phagocytosed bacteria (with rhodamine labelled antibody) hopefully to show that the lysozyme was surrounding the bacteria. The results of these experiments were suggestive of this state but not definite since the rhodamine that was used gave very poor fluorescence.

When slides of macrophages were stained after having phagocytosed bacteria, there were not the marked changes that were seen with the polymorphs; however, in some macrophages there was intense localizaed staining in the periphery of the cell which would correspond to the localization of the acid phosphatase that Cohn showed about injested particles. Thus, it is possible that in macrophages also there is a concentration of lysozyme within the phagocytic vacuoles.

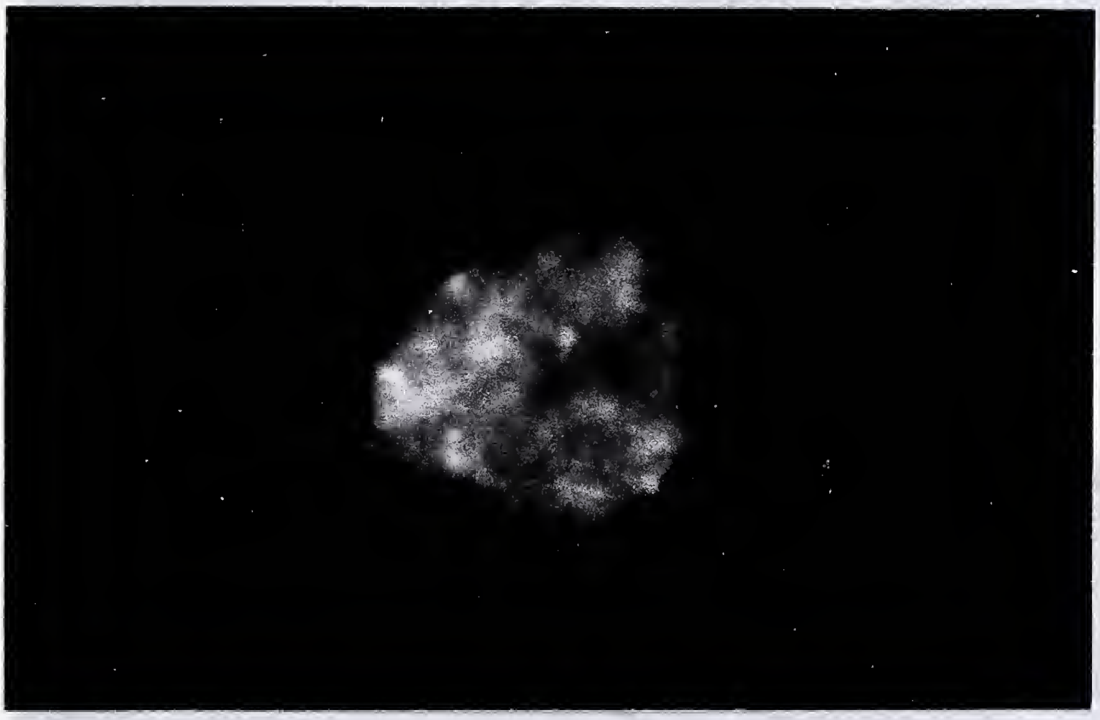


Plate 2

Mouse blood polymorphonuclear leucocyte, stained with rabbit anti-rat lysozyme serum and fluorescent anti-rabbit globulin.

x 300

Plate 3

Rat peritoneal exudate macrophages, stained as in Plate 2.

x 300

Plate 4

Mouse peritoneal exudate macrophages, stained as in Plate 2.

x 300

Plates 5-10

Polymorphonuclear leucocytes produced by the injection of dead *E. coli* in broth intraperitoneally in rats, stained as in Plate 2.

x 300

Note the apparent increase in size and eventual fusion of the lysozyme granules.

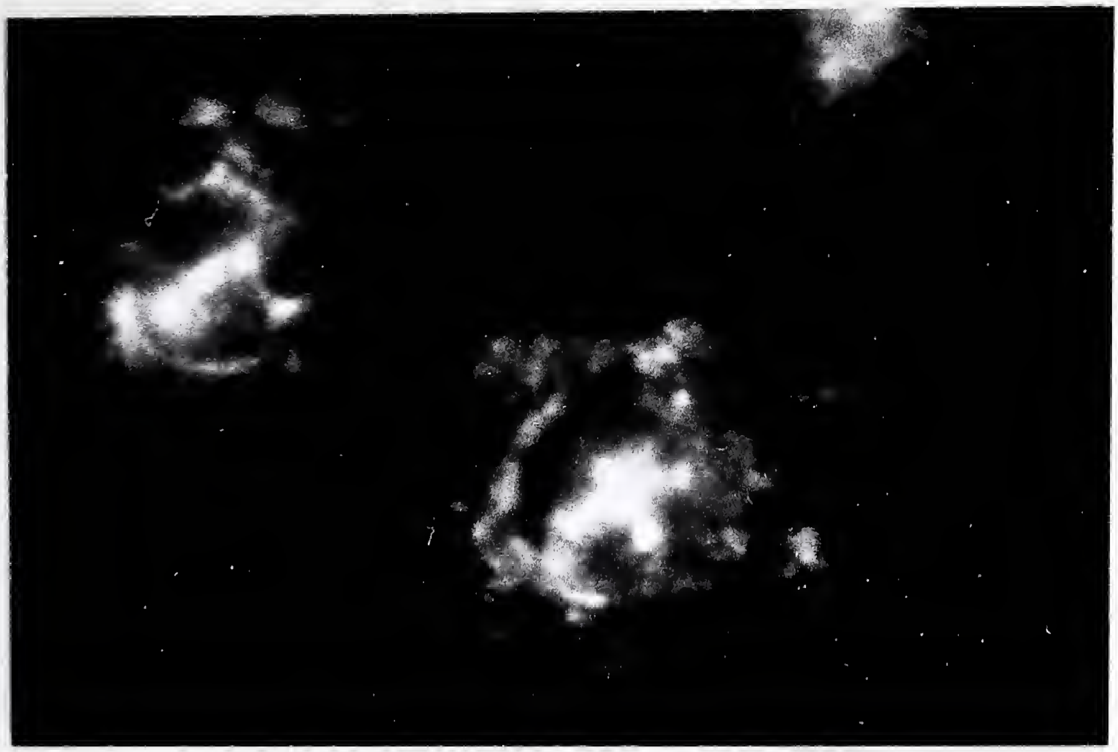


Plate 5

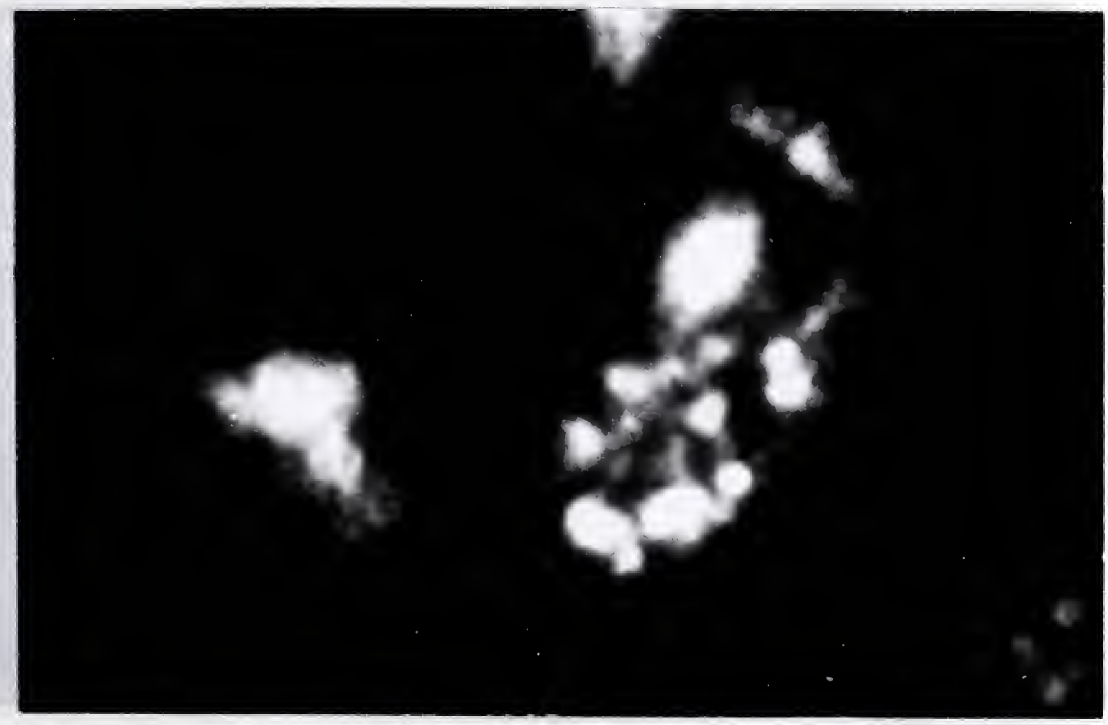


Plate 6

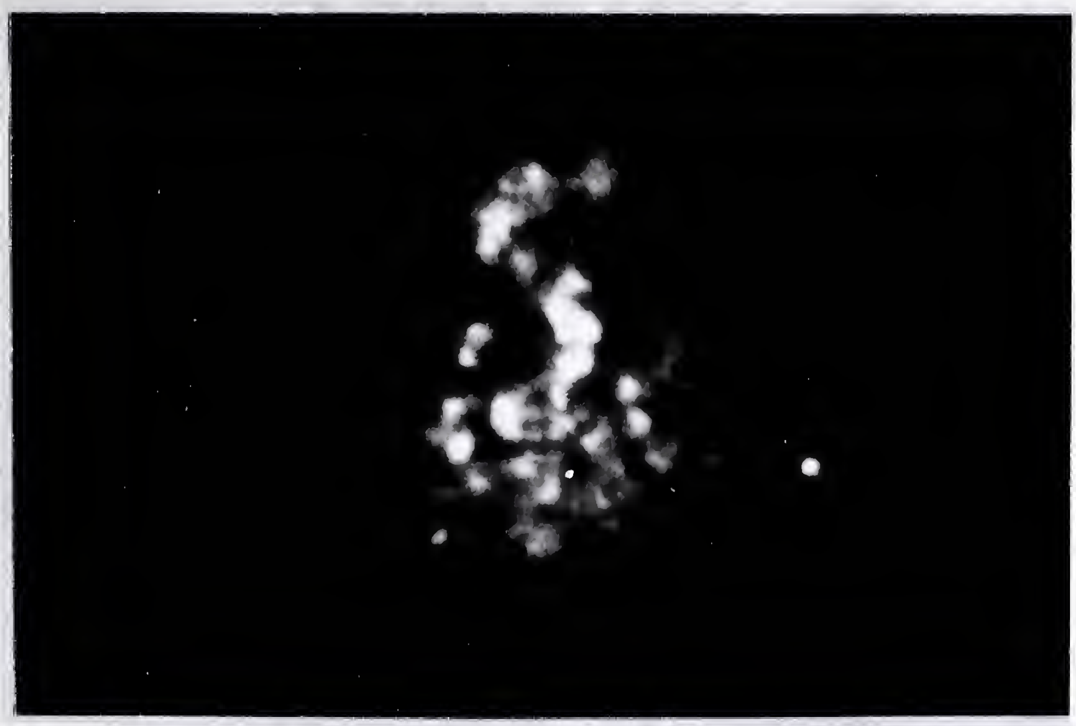


Plate 7

Plate 8

Plate 9

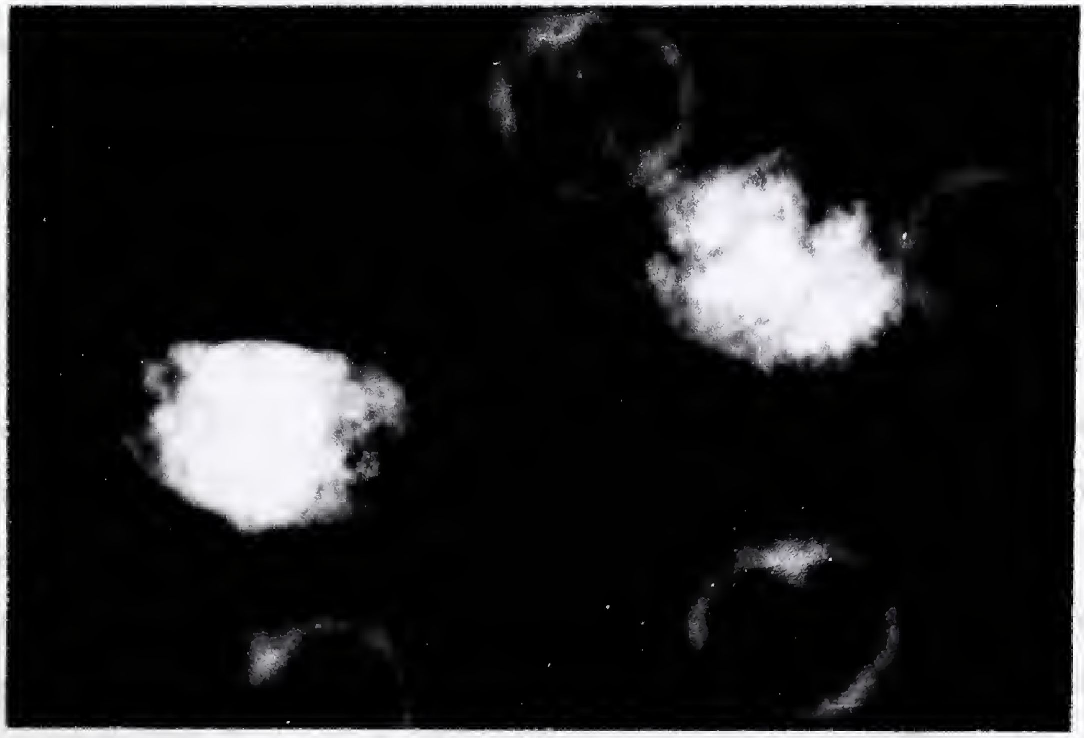


Plate 10

KIDNEY LYSOZYME ACTIVITY

History

Although Fleming's original work showed that the highest concentration of lysozyme was found in human tears, other work has shown that the highest concentration in most animal species is found in the kidneys (35,63,81). This finding is especially surprising since lysozyme is generally associated with the reticuloendothelial system and since the kidney is not usually accepted as a major component of the system. Increased lysozyme activity is found in most instances of increased RES activity: chronic infections (88), tumor implants (80), BCG infections, and polymorphonuclear leucocyte and macrophage concentration (29, 8). If lysozyme's only action is that of attacking the mucopolysaccharide backbone of sensitive bacteria, it is surprising that increases are found in the kidney lysozyme concentrations in states of RES stimulation.

Litwack in a series of papers (64, 66, 67) tried to show that the increase in kidney lysozyme activity was under the control of a pituitary-thyroid axis and was the result of endogenous renal production. He showed that _in vitro_ L-thyroxine and tri-iodo-L-tyrosine inhibited lysozyme's lytic activity while DL-thyroxine and di-iodo-L-tyrosine had no inhibitory effect. Further hypothysectomized rats had higher kidney levels of lysozyme than normal controls. He, therefore, felt that the endogenous kidney production of lysozyme was under the inhibitory control of the pituitary.

Perri, however, was able to show that the increase in the kidney lysozyme concentration was due to exogenous and not due to endogenous production (79, 82). He injected egg white lysozyme intravenously into rats, collected their urine for one hour, and then sacrificed them. Sixty

eight per cent of the injected lysozyme was recovered from the urine. There was a great increase in the lysozyme activity of the kidneys; however, the activity could be separated into two components by column chromatography. These fractions were shown to correspond to egg white lysozyme and rat kidney lysozyme, and, thus, 78% of the injected lysozyme could be accounted for. Perri, therefore, felt that the increased lysozyme concentrations that were found in the kidneys in states of RES stimulation were due to the accumulation of lysozyme rather than its endogenous production.

It has been shown many times that in normal urine of either humans or rats lysozyme is either absent or present in very small quantities, 0-2 ug/ml (78, 87, 135). However, in some renal diseases there is marked elevation of urinary lysozyme, lysozymuria. Several investigators have studied urinary lysozyme levels in attempts to gain data of prognostic value for persons suffering from the nephrotic syndrome (10, 136). Their conclusions, however, were that there was no correlation between the level of proteinuria and the degree of lysozymuria and that the lysozymuria was of no prognostic value.

More recent work has shown that increased urinary lysozyme levels are probably more a function of specific tubular damage rather than glomerular damage (87). Lysozyme because of its low molecular weight is filtered through the glomeruli and then is re-absorbed in the middle third of the proximal tubules. In cases of increased glomerular filtration no lysozymuria occurs unless the threshold of the tubules for lysozyme is exceeded due to increased blood levels or due to tubular damage. The production of the nephrotic syndrome experimentally in rats did not produce lysozymuria unless there was also treatment with mercuric chloride which produces tubular damage.

Method

Normal albino Wistar rats were injected intraperitoneally with 1 ml of incomplete Freund's adjuvant. After one month the Freund injected rats and normal rats were killed. Their kidneys were frozen, and cryostat sections (3 mu) made. These sections were placed on chemically cleaned slides and fixed in 95% ethanol. The sections were stained the same day with RB 200 labelled rabbit anti-rat kidney lysozyme with controls of immune and normal γ-globulin. These sections were mounted as usual and viewed under darkground fluorescent microscopy.

Discussion

In the normal kidney discrete regions of staining are seen in specific tubules near the glomeruli. These observations agree with those of Oliver <u>et al</u>. (75) who showed that the re-absorption of dilute protein solutions was accomplished by the formation of "droplets" in the middle third of the proximal tubules (118). (Plate 11-16).

The staining of the kidneys from the Freund's adjuvant stimulated rats shows a much more diffuse and much more generalized staining of the tubular cells. It has been shown that the lysozyme content of kidneys from BCG stimulated rats is twenty times that of normal kidneys (42). Oliver showed that, when the level of protein presented to the tubular cells is massive, there is not droplet formation with re-absorption, rather the re-absorbed protein is found diffusely throughout the tubules. Studies with the fluorescent antibody technique indicate that the concepts hold for lsyzoyme. (Plate 17-18).

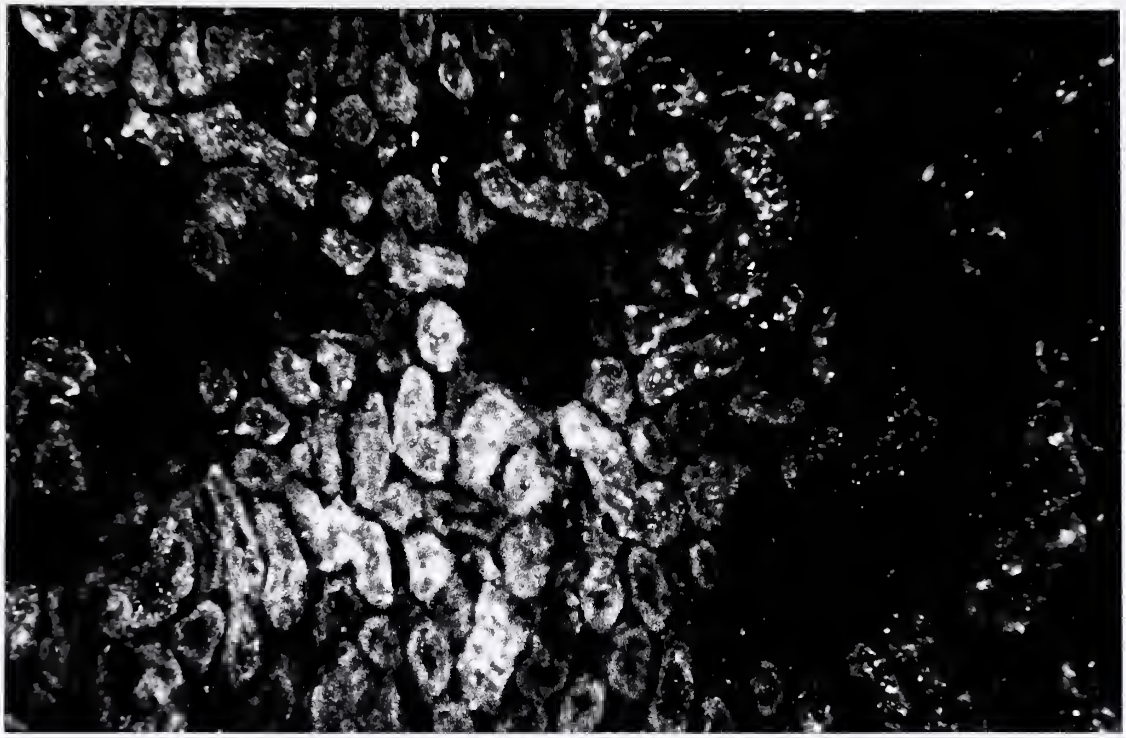


Plate 11

Normal rat kidney, stained with RB 200 labelled rabbit anti-rat lysozyme globulin.

x 60

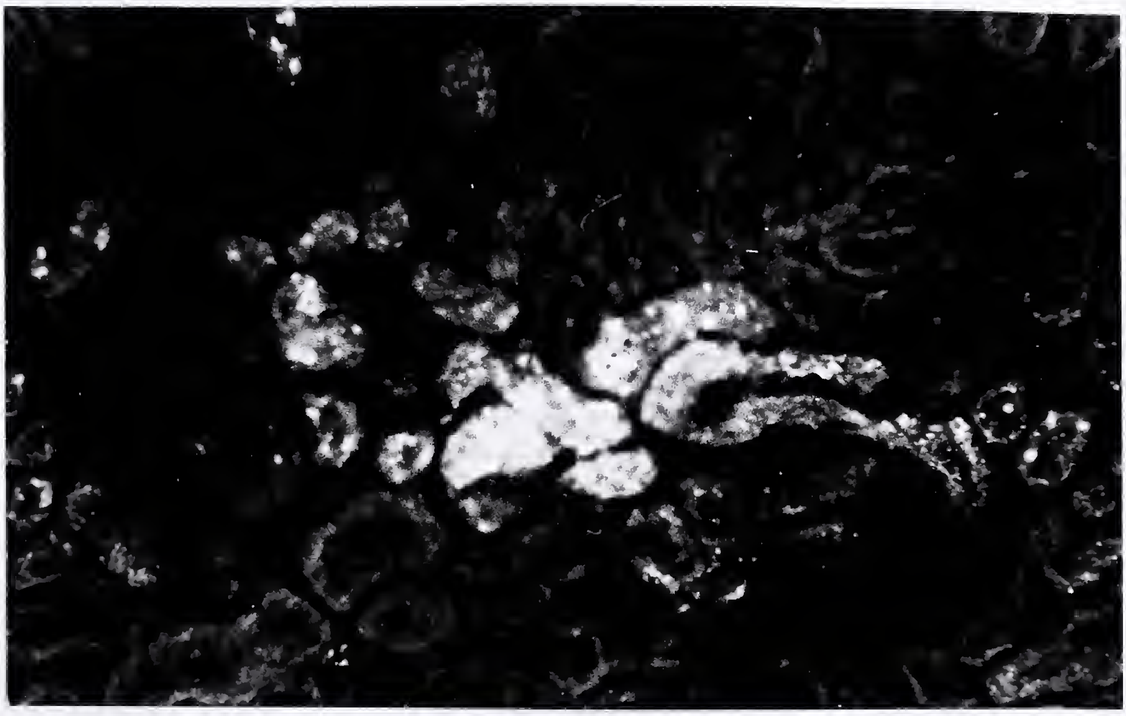


Plate 12

Normal rat kidney, stained as in Plate 11.

x 60

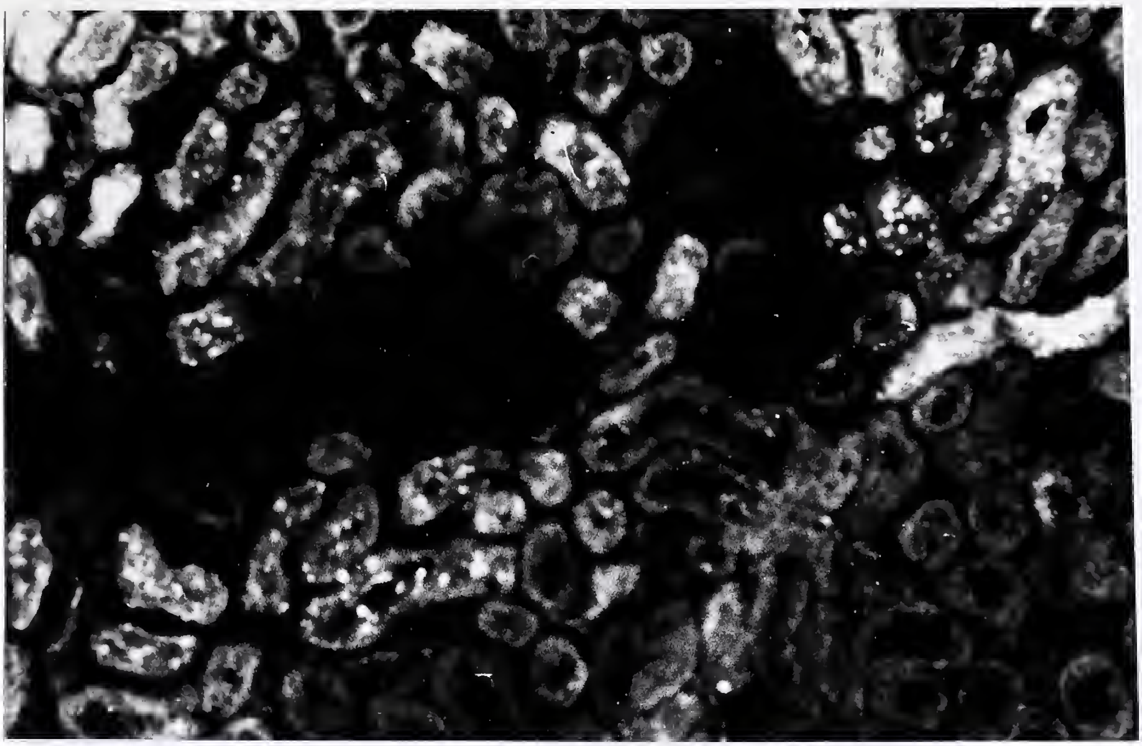


Plate 13

Normal rat kidney, stained as in Plate 11, normal γ-globulin control.

x 60

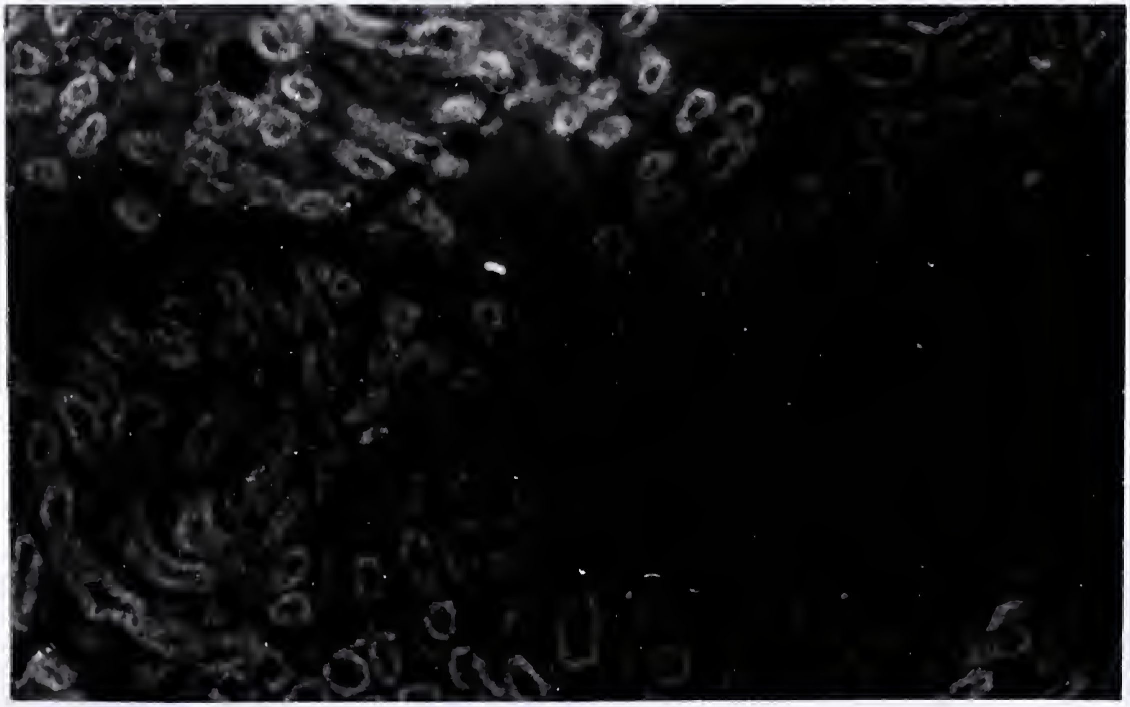


Plate 14

Normal rat kidney, stained as in Plate 11, immune γ-globulin control.

x 60

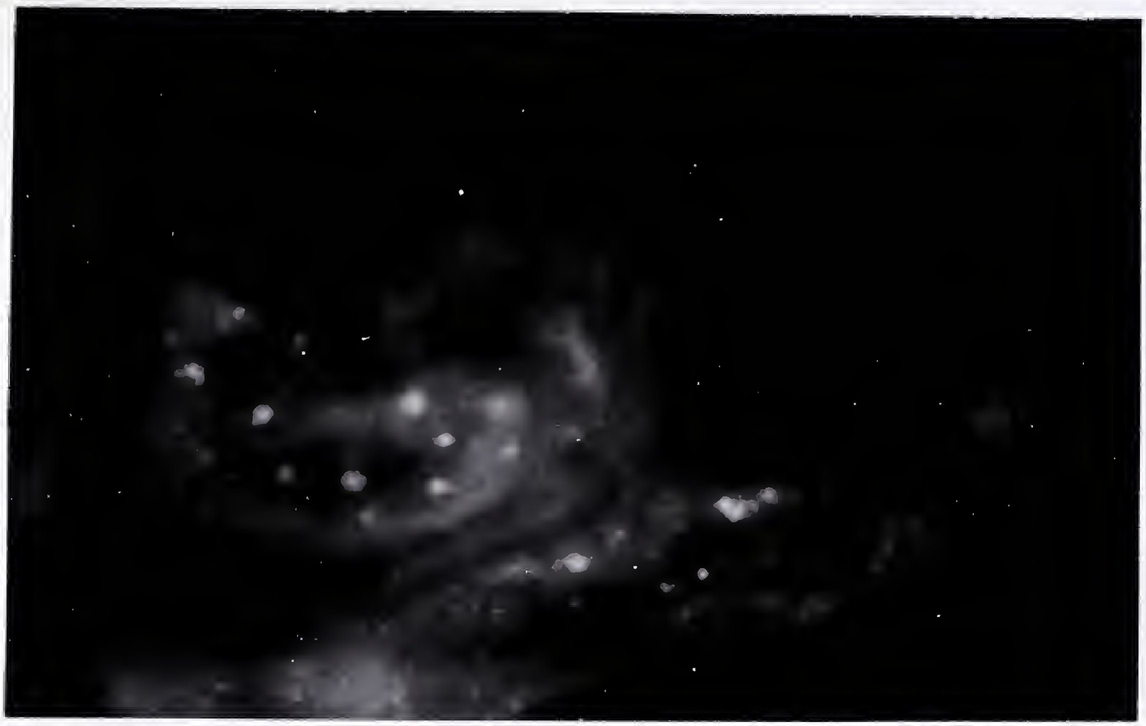


Plate 15

Normal rat kidney, stained as in Plate 11.

x 300

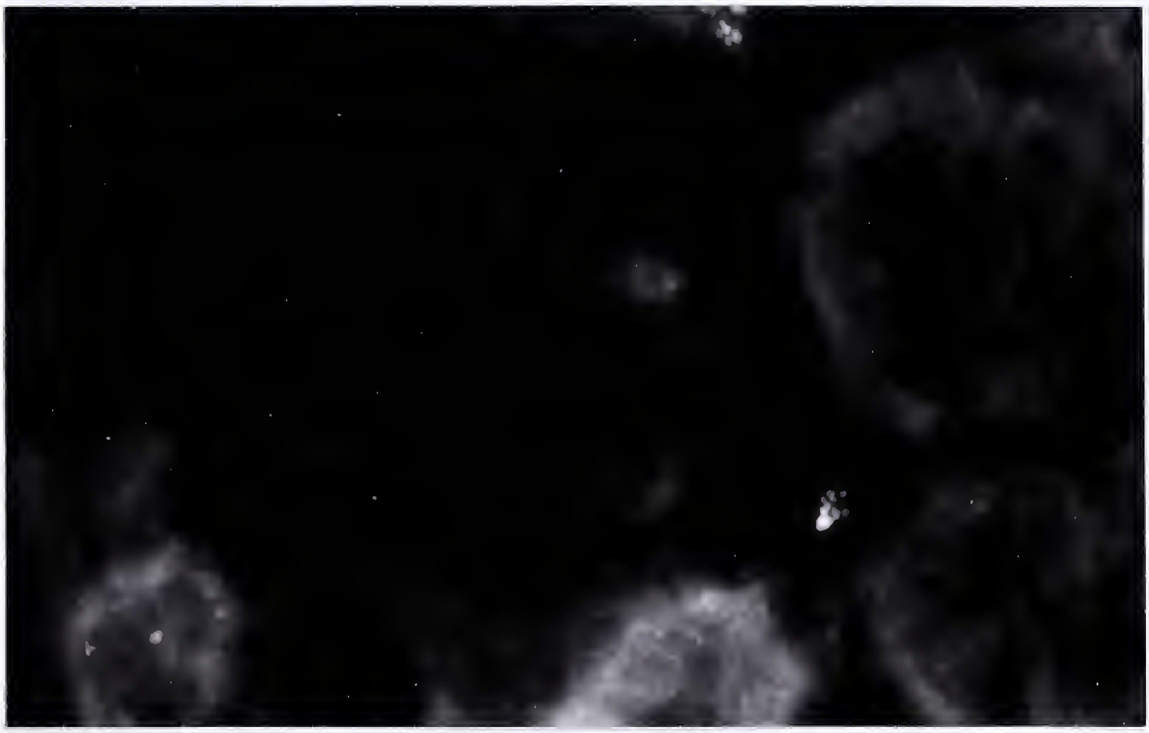


Plate 16

Normal rat kidney, stained as in Plate 11, immune γ-globulin control.

x 300

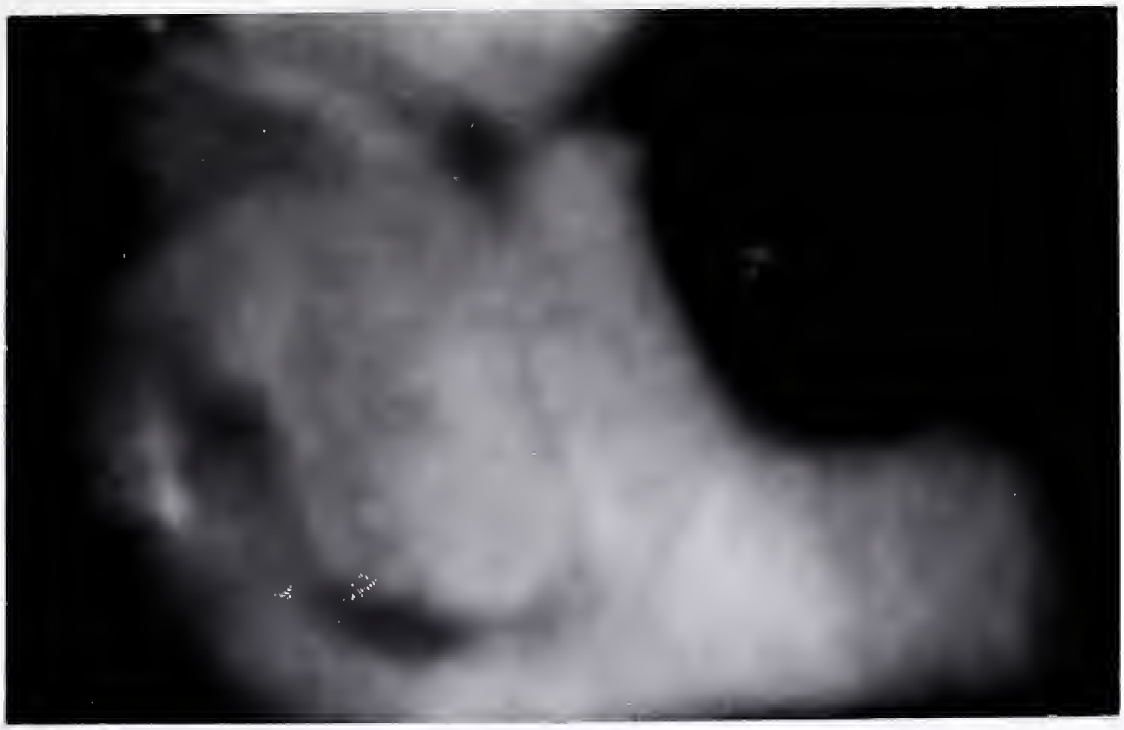


Plate 17

Freund's adjuvant stimulated rat kidney, stained as in Plate 11.

x 300

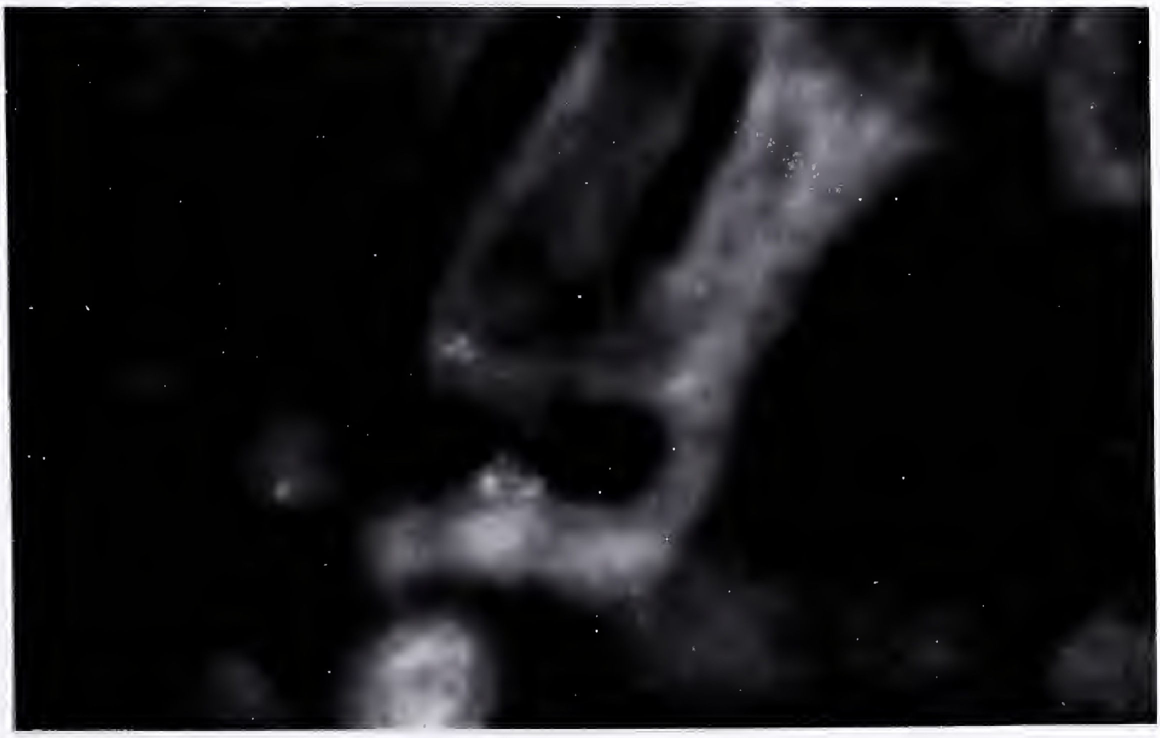


Plate 18

Freund's adjuvant stimulated rat kidney, stained as in Plate 11, immune γ-globulin control.

x 300

THE ENZYMATIC ACTION OF LYSOZYME

Introduction

Any enzymatic degradation can be divided into two steps: the formation of the enzyme-substrate complex and the subsequent chemical transformation of the complex (46). The chemical nature of the bond combining the substrate and the enzyme may be either covalent or ionic. The site of the binding also may be the site of the enzymatic activity of the enzyme. More likely, however, the combining site and the active site are physically separated. The combining site assures that the substrate is in the correct position so that the active site is in the proper position to react with the substrate.

Freeden (37), by means of methylation of lysozyme, was able to destroy its enzymatic activity but not to alter its ability to combine with substrate. This suggested that for lysozyme there is separation of combining and active sites. Fujio and his co-workers (38, 39, 115) showed even more clearly the separation of the combining and active sites. The production of antibodies in rabbits to purified egg white lysozyme produced two types of neutralizing antibodies. If the anti-egg white lysozyme was absorbed by the methyl ester of lysozyme, even with great antigen excess, 20% of the neutralizing antibody was not precipitated. All of it was precipitated, however, after unaltered egg white lysozyme was added. These results suggest there are two antigenic sites on the lysozyme molecule: one is or is close to the combining site and the second is or is close to the active site. The esterification of egg white lysozyme removes the second antigenic site but leaves the first unaltered. From this work it was determined that the valence of lysozyme was 5.

The efficiency of lysozyme is dependent upon the integrity of both its combining site and its active site. Alteration in either of these will alter the efficiency. It is not known if at the combining site of lysozyme whether the bond between the enzyme and the substrate is ionic or covalent. There is some evidence, however, that it is ionic, at least in the initial stages. It is natural to assume that there is some ionic component due to the high isoelectric point of lysozyme, 10.5-11.0, and, therefore, there will be some electrostatic forces between lysozyme and all substances with more physological isoelectric points. Moreover, the methylation product has even a high isoelectric point and combines even more avidly than lysozyme itself with the substrate. The change in isoelectric point may be the result of increased negativity, primarily in the region of the combining site. The increase in negative charge would increase the van der Waal's forces and, thus, the avidity of the bond.

The same situation can be produced by increasing the acidity of the substrate. Thus, the rate of the reaction between lysozyme and purified M. lysodeikitus cell walls is increased after the cell walls have been succinylated(111). This could be due to the increased van der Waal's forces between the substrate and the enzyme due to an increased charge difference.

It is more difficult to study alterations on the active site as opposed to the combining site since any alteration in the site will reduce or destroy enzymatic reactivity. The only chemical bond that lysozyme attacks is the β $(1 \rightarrow 4)$ bond of the AMA-AG disaccharide. Observed differences in reaction rates with different substrates are determined by the steric hindrance that the enzyme must overcome. Thus, lysozyme reacts more rapidly with the polysaccharide backbone than it does with the backbone with attached peptide chains.

Many investigators have studied the effect of chemical alteration of the lysozyme molecule on the action of the enzyme. Most of these have had no effect or have completely destroyed enzymatic activity; a very few have given graded response. One chemical alteration that is of interest is the oxidation of the tryptophan residues in lysozyme. There are six residues in the lysozyme molecule. They can be sequentially oxidized with a continual decrease in enzymatic activity until there is no activity when five of the residues have been oxidized (92). Since the valence of lysozyme is 5 and since the oxidation of each tryptophan residue reduces the activity by 20%, it is possible that each tryptophan residue forms part of active site amino acid sequence.

Experimental

The specific activity of an enzyme is the ratio between the activities of equal weights of the different forms of the enzyme. After purified preparations of human kidney lysozyme and rat kidney lysozyme had been obtained, their specific activities were determined as compared to purified Armour egg white lysozyme. The relative activities were determined by the usual method and expressed as ug equivalent egg white lysozyme (EEWL)/ml while the protein concentrations were determined by the method of Lowry.

Table II

Specific activities of various lysozymes

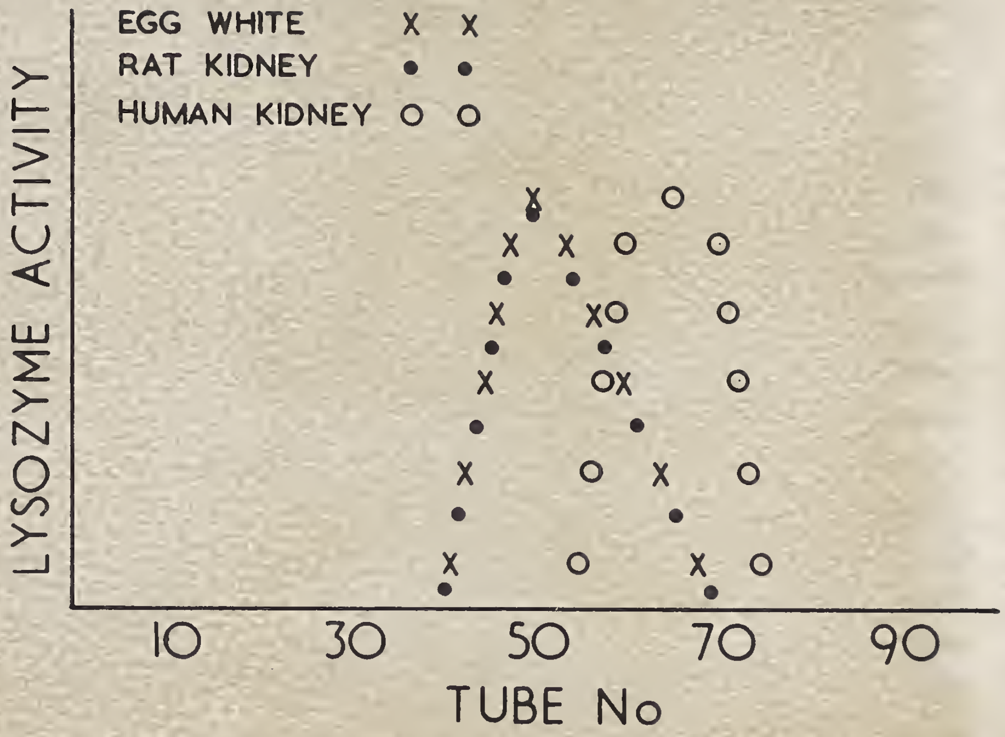
Source	Relative Activity ug EEWL/ml	Protein Concentration mg BSA/ml	Specific Activity
Egg White	100	24	1.00
Human Kidney I	62	8	1.85
Human Kidney II	89	12.5	1.71
Rat Kidney	75	20	.90

Discussion

The results are surprising since Jolles has reported (51) that human milk lysozyme is less active than egg white lysozyme. He showed that lysozyme preparations in decreasing order of specific activities were obtained from duck's eggs, hen eggs and human milk. This also is the order of the degree of their basic amino acid content. The fact that an increased basic amino acid content leads to a more active form of the enzyme is not surprising in view of the previous discussion.

The order of specific activity, human, egg, rat, agrees well, however, with the information that can be obtained from their elution from a CMC column by a pH-molar gradient (Figure 8). The egg white and rat kidney lysozymes are eluted over the same tube spread while the human kidney lysozyme is eluted at a higher pH and molarity, thus, demonstrating its greated basicity.

Attempts to confirm this order of specific activity by electrophoresis in acrylamide produced the order, egg, rat, human (Plate 19). In electrophoresis the separation is a function of the total molecular charge differences, and the separation on chromatographic columns is a function of the surface charge differences. It is not surprising that only the column chromatographic data agrees with the specific activities since the differences in activities are probably due to differences in surface charge rather than to differences in total charge. As was discussed, it appears to be the surface charge difference between the enzyme and the substrate which determines the rate of the reaction.



Relative positions of elution of different lysozymes

Figure 8

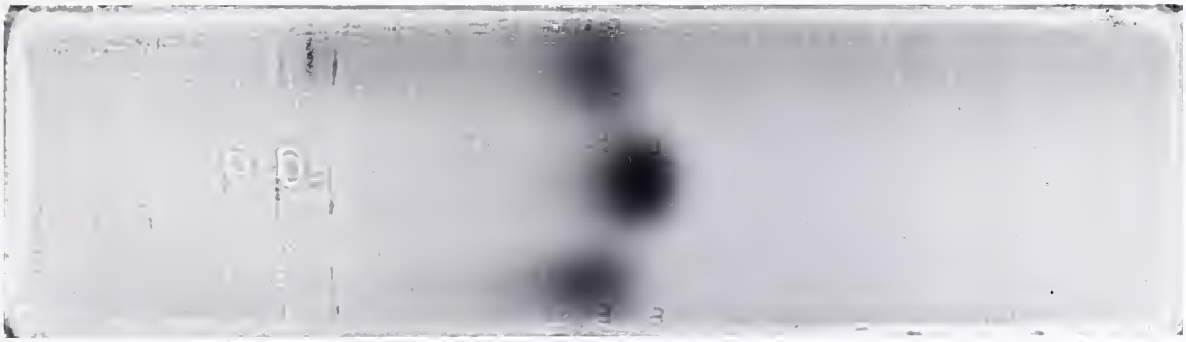


Plate 19

Electrophoresis of different lysozymes

5% acrylamide, barbitone buffer, pH 8.6, 18 hrs.

Top: Human Kidney Lysozyme

Middle: Purified Egg White Lysozyme

Bottom: Rat Kidney Lysozyme

ISOENZYMES

Introduction

Markert and Moller (71) defined isoenzymes as the different molecular forms in which proteins may exist with the same enzymatic specificity. In their work with lactic acid dehydrogenase they showed that five electrophoretically distinct forms existed and that the ratio of these forms varied with the source of the enzyme, even within the same species. The variation in the ratio of the different isoenzymes has lent itself to becoming a diagnostic tool. Whereas correlation of total serum LDH values with specific pathological changes is difficult, the correlation with specific isoenzyme changes is high. In heart miscle the electrophoretically faster components predominate while in liver and sketal muscle the slower components predominate. Thus, changes in the levels of the different isoenzymes of LDH is suggestive evidence of the site of disease (132).

It has been shown by column chromatography that egg white lysozyme can be separated into two enzymatically active components (1,2) and that there is an interconversion between these two components (124, 125). Attempts at their electrophoretic separation have not been definitive. One group reported the occasional presence of a second band (5, 126) while another reported the presence of one prinicpal peak with shoulders which they interpreted as hetrogenicity (89).

In the attempts to determine the relative electrophoretic mobilities of lysozyme from different sources, the separation of the isoenzymes of egg white lysozyme and the partial separation of the isoenzymes of human kidney lysozyme was achieved.

Method

A 5% solution of acrylamide in buffer was made up (Cyanogum 41, British Drug House) to which was added 0.13 ml 2-dimethyl-amino-ethyl cyanide per 100 ml of solution per 1% acrylamide. Then 0.13 ml of freshly prepared ammonium persulfate was added per 100 ml of solution per 1% acrylamide (93). The solution was poured into the mold and sealed. The rate of gelling could be hastened by placing the mold in an incubator at 37°C. A mold, 25 x 10 x 1 cm, was used; this size allowed the study of four samples at once. In some experiments the gel was made up with 8M urea in order to achieve better separation of the isoenzymes (116).

The power source used was a VOKAM power pack (Shandon Scientific Co., Ltd., London). The buffer system was discontinuous with the cathode bath at a high molarily so as to reverse the normal electro-osmotic flow. The protein solution under study were in the range, 5-10 mg/ml. They were applied to Whatman's #1 filter paper and placed in slits or were placed directly into slots in the gel. A constant amperage of 2.5 mA/cm was used (~150 V)., and the duration of the runs was between 12 and 25 hours. At the completion of a run the gel was fixed for 15 minutes in 10% TCA and then stained in a concentrated solution of Light Green stain. The gel was then washed in a 5% acetic acid-water until clear.

Discussion

There was separation of both Armour crude egg white and Armour purified egg white lysozymes into two electrophoretically distinct components. There

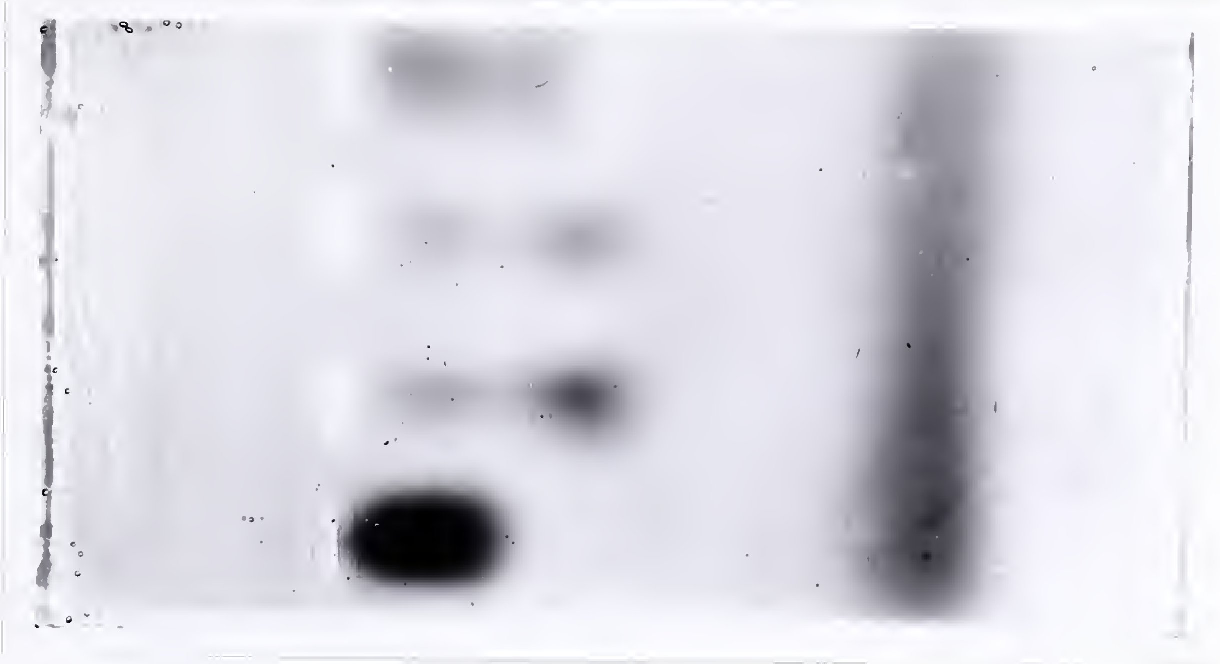


Plate 20

Electrophoresis of different lysozymes

4% acrylamide, 8M urea, glycine buffer, pH 10.3, 24 hrs.

Top:	Human Kidney Lysozyme
Upper Middle:	Purified Egg White Lysozyme
Lower Middle:	Crude Egg White Lysozyme
Bottom:	Rat Kidney Lysozyme

also was partial separation of human kidney lysozyme into two components (Plate 20). These results agree with the results from the column chromatography of egg white lysozyme (124, 125). The separation of freshly purified egg white lysozyme on IRC-50 yielded two peaks whose ratio was 95-5. Chromatography of the same sample after one year gave a ratio of 60-40 leading the authors to believe that there was an interconversion between the two forms. Densometric analysis of the separation products of the Armour purified egg white lysozyme in acrylamide showed a ratio of 88-12 while the Armour crude egg white lysozyme showed a ratio of 70-30. These values agree well with the column data. The question arises as to whether the purification process selectively removes one component or whether it causes an interconversion of the forms which reverts if the lysozyme is allowed to stand.

Litwack (68) noted the presence of two peaks of activity in his purification of human kidney lysozyme on IRC-50 but he was unable to obtain complete separation. The availability of newer techniques now should make possible the electrophoretic separation of human kidney lysozyme into its two components. In these studies no separation of rat kidney lysozyme occured although Litwack (65) has reported the presence of three peaks by column chromatography.

Whereas other enzymes besides LDH are being subjected to diagnostic isoenzyme separation, i.e. L-glutamate dehydrogenase (129), it is possible that the determination of the ratios of the isoenzymes of lysozyme from different human tissues might become a convenient diagnostic aid.

SUMMARY

A new method has been established for the extraction of lysozyme from human kidney. During the extraction procedure a 3½ fold increase in activity was achieved by the removal of an inhibitor, possible spermine.

The localization of lysozyme in rat and mouse phagocytic cells before and after the phagocytosis of dead bacteria has been studied be means of the indirect fluorescent antibody technique. Normal polymorphonuclear leucocytes showed locatization of the lysozyme in cytoplasmic granules with a faint, diffuse cytoplasmic staining while in the macrophages the lysozyme was diffusely distributed throughout the cytoplasm. After phagocytosis the polymorphonuclear leucocytes showed a variable pattern which included larger granules, crescent formation, and generalized cytoplasmic staining. In contrast, in the macrophages there was little visible change except for occasional cytoplasmic droplet formation showing localized concentration of lysozyme.

The localization of lysozyme by the fluorescent technique agrees wiith the enzymatic assay for lysozyme that have been done on these cells before and after phagocytosis by other authors. Several questions still remain unanswered. It still has not been shown that the concentration of the lysozyme that occurs after phagocytosis is in the phagocytic vacuoles. The proof of this concentration could be obtained either through double fluorescent antibody studies or with ferritin labelled anti-lysozyme and electron microscopy.

The reason why the anti-lysozyme was originally prepared was for the further study of the role of lysozyme in the intracellular destruction of bacteria. These studies could now be continued using a double antibody against both the bacteria under study and lysozyme hoping to show a differ-

ence in the killing curve between those bacteria protected with the anti-lysozyme and those unprotected. The difference between the killing curves would be a measure of the role of lysozyme in the destruction of that species of bacteria. These studies should cover a wide spectrum of bacteria to see what the role of lysozyme is in resistance to each of them.

In normal rat kidneys it was shown that lysozyme is found in droplets in the proximal tubules by the direct fluorescent antibody technqiue while in kidneys from Freund's adjuvant stimulated rats there was diffuse staining of all the tubular cells. The fate of lysozyme in the kidney is uncertain; is it enzymatically degraded and the resultant amino acids and peptides released into the blood stream or is it released as unaltered lysozyme that can be re-utilized by phagocytic cells without alteration. Answers to these questions would have applicability to the whole field of protein metabolism. Answers could be derived by I^{131} labelling of purified rat lysozyme and injecting it into rats. The majority of it would be re-absorbed by the kidney tubules, and then its ultimate distribution could be determined which would give evidence as to its metabolism. If the I^{131} was found only in phagocytic cells, then it is probably that the lysozyme is re-utilized in an unaltered state, but, if the label was found diffusely throughout the body, it would show that the lysozyme had undergone degradation.

The specific activities of a series of lysozymes from different sources was established: human kidney, 1.75, egg white, 1.00, and rat kidney, 0.90.

The electrophoretic separation in acrylamide of the isoenzymes of egg white lysozyme was achieved as well as the partial separation of the isoenzymes of human kidney lysozyme. There are two electrophoretically separatable compenents to both enzymes. Further work should be carried out on the human lysozyme to see if there is any organ specific pattern of ratio of the isoenzymes that might be a diagnostic aid in clinical medicine.

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